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(54) Title: METHODS AND REAGENTS FOR VACCINATION WHICH GENERATE A CD8 T CELL IMMUNE RESPONSE			
(57) Abstract			
<p>New methods and reagents for vaccination are described which generate a CD8 T cell immune response against malarial and other antigens such as viral and tumour antigens. Novel vaccination regimes are described which employ a priming composition and a boosting composition, the boosting composition comprising a non-replicating or replication-impaired pox virus vector carrying at least one CD8 T cell epitope which is also present in the priming composition.</p>			

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METHODS AND REAGENTS FOR VACCINATION WHICH GENERATE A CD8 T CELL IMMUNE RESPONSE

This invention relates to generation of a protective CD8+ T cell immune response against target antigens using different primer and
5 booster compositions as sources of CD8+ T cell epitopes.

Introduction

A general problem in vaccinology has been an inability to generate high levels of CD8 T cells by immunisation. This has impeded
10 the development of vaccines against several diseases including malaria.

Plasmodium falciparum malaria causes hundreds of millions of malaria infections each year and is responsible for 1-2 million deaths annually. The development of an effective vaccine against malaria is thus a major priority for global public health. A considerable body of
15 immunological research over the last twenty years had led to the identification both of candidate vaccine antigens from the parasite and immunological mechanisms on the host that are likely to protect against infection and disease. However, despite this progress there is still no means of vaccinating against malaria infection which has been shown to
20 be effective in field trials.

A major problem has been the identification of a means of inducing a sufficiently strong immune response in vaccinated individuals to protect against infection and disease. So, although many malaria antigens are known that might be useful in vaccinating against malaria the problem
25 has been how to deliver such antigens or fragments of them known as epitopes, which are recognised by cells of the immune system, in a way that induces a sufficiently strong immune response of a particular type.

It has been known for many years that it is possible to protect individuals by immunising them with very large doses of irradiated malaria
30 sporozoites given by bites from infected mosquitoes. Although this is a

wholly impractical method of mass vaccination it has provided a model for analysing the immune responses that might be mediating protective immunity against sporozoite infection (Nardin and Nussenzweig 1993).

A considerable amount of research over the last decade or
5 more has indicated that a major protective immune response against the early pre-erythrocytic stage of *P. falciparum* malaria is mediated by T lymphocytes of the CD8+ ve type (CD8+ T cells). Such cells have been shown to mediate protection directly in mouse models of malaria infection (Nardin and Nussenzweig 1993). Such T cells have also been identified in
10 individuals naturally exposed to malaria and in volunteers immunised with irradiated sporozoites (Hill *et al.* 1991; Aidoo *et al.* 1995; Wizen *et al.* 1995). There is much indirect evidence that such CD8+ T cells are protective against malaria infection and disease in humans (Lalvani *et al.* 1994).

CD8+ T cells may function in more than one way. The best
15 known function is the killing or lysis of target cells bearing peptide antigen in the context of an MHC class I molecule. Hence these cells are often termed cytotoxic T lymphocytes (CTL). However, another function, perhaps of greater protective relevance in malaria infections is the ability of CD8+ T cells to secrete interferon gamma (IFN- γ). Thus assays of lytic activity and
20 of IFN- γ release are both of value in measuring a CD8+ T cell immune response. In malaria these CD8+ve cells can protect by killing the parasite at the early intrahepatic stage of malaria infection before any symptoms of disease are produced (Seguin *et al.* 1994).

The agent of fatal human malaria, *P. falciparum* infects a
25 restricted number of host species: humans, chimpanzees and some species of New World monkey. The best non-human model of malaria is the chimpanzee because this species is closely related to humans and liver-stage infection is observed consistently unlike in the monkey hosts (Thomas *et al.* 1994). Because of the expense and limited availability of
30 chimpanzees most laboratory studies of malaria are performed in mice,

using the rodent malaria species *P. berghei* or *P. yoelii*. These latter two models are well studied and it has been shown in both that CD8+ve lymphocytes play a key role in protective immunity against sporozoite challenge.

5 Previous studies have assessed a large variety of means of inducing CD8+ T cell responses against malaria. Several of these have shown some level of CD8+ T cell response and partial protection against malaria infection in the rodent models (e.g. Li *et al.* 1993; Sedegah *et al* 1994; Lanar *et al.* 1996). However, an effective means of immunising with
10 subunit vaccines by the induction of sufficiently high levels of CD8+ T lymphocytes to protect effectively against malaria sporozoite infection has not previously been demonstrated.

 In recent years improved immune responses generated to potential vaccines have been sought by varying the vectors used to deliver
15 the antigen. There is evidence that in some instances antibody responses are improved by using two different vectors administered sequentially as prime and boost. A variety of combinations of prime and boost have been tested in different potential vaccine regimes.

 Leong *et al.* (Vaccines 1995, 327-331) describe immunising
20 mice firstly to DNA expressing the influenza haemagglutinin (HA) antigen and then with a recombinant fowlpox vector expressing HA. An enhanced antibody response was obtained following boosting.

 Richmond *et al.* (Virology 1997, 230: 265-274) describe attempts to raise neutralising antibodies against HIV-1 env using DNA
25 priming and recombinant vaccinia virus boosting. Only low levels of antibody responses were observed with this prime boost regime and the results were considered disappointing.

 Fuller *et al.* (Vaccine 1997, 15:924-926 and Immunol Cell Biol 1997, 75:389-396) describe an enhancement of antibody responses to
30 DNA immunisation of macaques by using a booster immunisation with

replicating recombinant vaccinia viruses. However, this did not translate into enhanced protective efficacy as a greater reduction in viral burden and attenuation of CD4 T cell loss was seen in the DNA primed and boosted animals.

5 Hodge *et al* (Vaccine 1997, 15: 759-768) describe the induction of lymphoproliferative T cell responses in a mouse model for cancer using human carcinoembryonic antigen (CEA) expressed in a recombinant fowl pox virus (ALVAC). The authors primed an immune response with CEA-recombinant replication competent vaccinia viruses of
10 the Wyeth or WR strain and boosted the response with CEA-recombinant ALVAC. This led to an increase in T cell proliferation but did not result in enhanced protective efficacy if compared to three wild type recombinant immunisations (100% protection), three recombinant ALVAC-CEA immunisations (70% protection) or WR prime followed by two ALVAC-CEA
15 immunisations (63% protection).

 Thus some studies of heterologous prime-boost combination have found some enhancement of antibody and lymphoproliferative responses but no significant effect on protective efficacy in an animal model. CD8 T cells were not measured in these studies. The limited
20 enhancement of antibody response probably simply reflects the fact that antibodies to the priming immunogen will often reduce the immunogenicity of a second immunisation with the same immunogen, while boosting with a different carrier will in part overcome this problem. This mechanism would not be expected to be significantly affected by the order of immunisation.

25 Evidence that a heterologous prime boost immunisation regime might affect CD8 T cell responses was provided by Li *et al.* (1993). They described partial protective efficacy induced in mice against malaria sporozoite challenge by administering two live viral vectors, a recombinant replicating influenza virus followed by a recombinant replicating vaccinia
30 virus encoding a malaria epitope. Reversing the order of immunisation led

to loss of all protective efficacy and the authors suggested that this might be related to infection of liver cells by vaccinia, resulting in localisation of CTLs in the liver to protect against the hepatocytic stages of malaria parasites.

5 Rodrigues *et al.* (J. Immunol. 1994, 4636-4648) describe immunising mice with repeated doses of a recombinant influenza virus expressing an immunodominant B cell epitope of the malarial circumsporozoite (CS) protein followed by a recombinant vaccinia virus booster. The use of a wild type vaccinia strain and an attenuated but
10 replication-competent vaccinia strain in the booster yielded very similar levels of partial protection. However the attenuated but replication competent strain was slightly less immunogenic for priming CD8 T cells than the wild type vaccinia strain.

 Murata *et al.* (Cell. Immunol. 1996, 173: 96-107) reported
15 enhanced CD8 T cell responses after priming with replicating recombinant influenza viruses and boosting with a replicating strain of vaccinia virus and suggested that the partial protection observed in the two earlier studies was attributable to this enhanced CD8 T cell induction.

 Thus these three studies together provide evidence that a
20 booster immunisation with a replicating recombinant vaccinia virus may enhance to some degree CD8 T cell induction following priming with a replicating recombinant influenza virus. However, there are two limitations to these findings in terms of their potential usefulness. Firstly, the immunogenicity induced was only sufficient to achieve partial protection
25 against malaria and even this was dependent on a highly immunogenic priming immunisation with an unusual replicating recombinant influenza virus. Secondly, because of the potential and documented side-effects of using these replicating viruses as immunogens these recombinant vectors are not suitable for general human use as vaccines.

Modified vaccinia virus Ankara (MVA) is a strain of vaccinia virus which does not replicate in most cell types, including normal human tissues. MVA was derived by serial passage >500 times in chick embryo fibroblasts (CEF) of material derived from a pox lesion on a horse in
5 Ankara, Turkey (Mayr *et al.* 1975). It was shown to be replication-impaired yet able to induce protective immunity against veterinary poxvirus infections (Mayr 1976). MVA was used as a human vaccine in the final stages of the smallpox eradication campaign, being administered by intracutaneous, subcutaneous and intramuscular routes to >120,000
10 subjects in southern Germany. No significant side effects were recorded, despite the deliberate targeting of vaccination to high risk groups such as those with eczema (Mayr *et al.* 1978; Stickl *et al.* 1974; Mahnel *et al.* 1994;). The safety of MVA reflects the avirulence of the virus in animal models, including irradiated mice and following intracranial administration
15 to neonatal mice. The non-replication of MVA has been correlated with the production of proliferative white plaques on chick chorioallantoic membrane, abortive infection of non-avian cells, and the presence of six genomic deletions totalling approximately 30 kb (Meyer *et al.* 1991). The avirulence of MVA has been ascribed partially to deletions affecting host
20 range genes K1L and C7L, although limited viral replication still occurs on human TK-143 cells and African Green Monkey CV-1 cells (Altenburger *et al.* 1989). Restoration of the K1L gene only partially restores MVA host range (Sutter *et al.* 1994). The host range restriction appears to occur during viral particle maturation, with only immature virions being observed
25 in human HeLa cells on electron microscopy (Sutter *et al.* 1992). The late block in viral replication does not prevent efficient expression of recombinant genes in MVA. Recombinant MVA expressing influenza nucleoprotein, influenza haemagglutinin, and SIV proteins have proved to be immunogenic and provide varying degrees of protection in animal
30 models, although this has never been ascribed to CD8+ T lymphocytes

alone (Sutter *et al.* 1994, Hirsch *et al.* 1995; Hirsch *et al.* 1996).

Recombinant MVA is considered a promising human vaccine candidate because of these properties of safety and immunogenicity (Moss *et al.* 1995). Recombinant MVA containing DNA which codes for foreign
5 antigens is described in US 5,185,146 (Altenburger).

Poxviruses have evolved strategies for evasion of the host immune response that include the production of secreted proteins that function as soluble receptors for tumour necrosis factor, IL-1 β , interferon (IFN)- α/β and IFN- γ , which normally have sequence similarity to the
10 extracellular domain of cellular cytokine receptors (Symons *et al.* 1995; Alcamí *et al.* 1995; Alcamí *et al.* 1992). The most recently described receptor of this nature is a chemokine receptor (Graham *et al.* 1997). These viral receptors generally inhibit or subvert an appropriate host immune response, and their presence is associated with increased
15 pathogenicity. The IL-1 β receptor is an exception: its presence diminishes the host febrile response and enhances host survival in the face of infection (Alcamí *et al.* 1996). We have discovered that MVA lacks functional cytokine receptors for interferon γ , interferon $\alpha\beta$, Tumour Necrosis Factor and CC chemokines, but it does possess the potentially
20 beneficial IL-1 β receptor. MVA is the only known strain of vaccinia to possess this cytokine receptor profile, which theoretically renders it safer and more immunogenic than other poxviruses. Another replication-impaired and safe strain of vaccinia known as NYVAC is fully described in Tartaglia *et al.* (Virology 1992, 188: 217-232).

25 It has long been recognised that live viruses have some attractive features as recombinant vaccine vectors including a high capacity for foreign antigens and fairly good immunogenicity for cellular immune responses (Ellis 1988 new technologies for making vaccines. In: Vaccines. Editors: Plotkin S A and Mortimer E A. W B Saunders,
30 Philadelphia, page 568; Woodrow G C 1977. In: New Generation

Vacciness 2nd Edition. Editors: Levine M M, Woodrow G C, Kaper J B, Cobon G, page 33). This has led to attempts to attenuate the virulence of such live vectors in various ways including reducing their replication capacity (Tartaglia J *et al.* 1992 Virology 188: 217-232). However such a
5 reduction in replication reduces the amount of antigen produced by the virus and thereby would be expected to reduce vaccine immunogenicity. Indeed attenuation of replicating vaccinia strains has previously been shown to lead to some substantial reductions in antibody responses (Lee M S *et al.*, 1992 J Virology 66: 2617-2630). Similarly the non-replicating
10 fowlpox vector was found to be less immunogenic for antibody production and less protective than a replicating wild-type vaccinia strain in a rabies study (Taylor J *et al.* 1991 Vaccine 9: 190-193).

It has now been discovered that non-replicating and replication-impaired strains of poxvirus provide vectors which give an
15 extremely good boosting effect to a primed CTL response. Remarkably, this effect is significantly stronger than a boosting effect by wild type poxviruses. The effect is observed with malarial and other antigens such as viral and tumour antigens, and is protective as shown in mice and non-human primate challenge experiments. Complete rather than partial
20 protection from sporozoite challenge has been observed with the novel immunisation regime.

It is an aim of this invention to identify an effective means of immunising against malaria. It is a further aim of this invention to identify means of immunising against other diseases in which CD8+ T cell
25 responses play a protective role. Such diseases include but are not limited to infection and disease caused by the viruses HIV, herpes simplex, herpes zoster, hepatitis C, hepatitis B, influenza, Epstein-Barr virus, measles, dengue and HTLV-1; by the bacteria *Mycobacterium tuberculosis* and *Listeria* sp.; and by the protozoan parasites *Toxoplasma* and

Trypanosoma; and certain forms of cancer e.g. melanoma, cancer of the breast and cancer of the colon.

We describe here a novel method of immunising that generated very high levels of CD8+ T cells and was found to be capable of inducing unprecedented complete protection against *P. berghei* sporozoite challenge. The same approach was tested in higher primates and found to be highly immunogenic in this species also, and was found to induce partial protection against *P. falciparum* challenge. Induction of protective immune responses has also been demonstrated in two additional mouse models of viral infection and cancer.

We show further than the novel immunisation regime that is described here is also effective in generating strong CD8+ T cell responses against HIV epitopes. Considerable evidence indicates that the generation of such CD8+ T cell responses can be expected to be of value in prophylactic or therapeutic immunisation against this viral infection and disease (Gallimore *et al* 1995; Ada 1996). We demonstrate that strong CD8+T cell responses may be generated against epitopes from both HIV and malaria using an epitope string with sequences from both of these micro-organisms. The success in generating enhanced immunogenicity against both HIV and malaria epitopes, and also against influenza and tumour epitopes, indicates that this novel immunisation regime can be effective generally against many infectious pathogens and also in non-infectious diseases where the generation of a strong CD8+ T cell response may be of value.

A surprising feature of the current invention is the finding of the very high efficacy of non-replicating agents in both priming and particularly in boosting a CD8+ T cell response. In general the immunogenicity of CD8+ T cell induction by live replicating viral vectors has previously been found to be higher than for non-replicating agents or replication-impaired vectors. This is as would be expected from the greater

amount of antigen produced by agents that can replicate in the host. Here however we find that the greatest immunogenicity and protective efficacy is surprisingly observed with non-replicating vectors. The latter have an added advantage for vaccination in that they are in general safer for use in humans than replicating vectors.

The present invention provides in one aspect a kit for generating a protective CD8+ T cell immune response against at least one target antigen, which kit comprises:

- (i) a priming composition comprising a source of one or more CD8+ T cell epitopes of the target antigen, together with a pharmaceutically acceptable carrier; and
- (ii) a boosting composition comprising a source of one or more CD8+ T cell epitopes of the target antigen, including at least one CD8+ T cell epitope which is the same as a CD8+ T cell epitope of the priming composition, wherein the source of CD8+ T cell epitopes is a non-replicating or replication-impaired recombinant poxvirus vector, together with a pharmaceutically acceptable carrier;

with the proviso that if the source of epitopes in (i) is a viral vector, the viral vector in (ii) is derived from a different virus.

In another aspect the invention provides a method for generating a protective CD8+ T cell immune response against at least one target antigen, which method comprises administering at least one dose of component (i), followed by at least one dose of component (ii) of the kit according to the invention.

Preferably, the source of CD8+ T cell epitopes in (i) in the method according to the invention is a non-viral vector or a non-replicating or replication-impaired viral vector, although replicating viral vectors may be used.

Preferably, the source of CD8+ T cell epitopes in (i) is not a poxvirus vector, so that there is minimal cross-reactivity between the primer and the booster.

In one preferred embodiment of the invention, the source of
5 CD8+ T cell epitopes in the priming composition is a nucleic acid, which may be DNA or RNA, in particular a recombinant DNA plasmid. The DNA or RNA may be packaged, for example in a lysosome, or it may be in free form.

In another preferred embodiment of the invention, the source
10 of CD8+ T cell epitopes in the priming composition is a peptide, polypeptide, protein, polyprotein or particle comprising two or more CD8+ T cell epitopes, present in a recombinant string of CD8+ T cell epitopes or in a target antigen. Polyproteins include two or more proteins which may be the same, or preferably different, linked together. Particularly preferred in
15 this embodiment is a recombinant proteinaceous particle such as a Ty virus-like particle (VLP) (Burns *et al.* Molec. Biotechnol. 1994, 1: 137-145).

Preferably, the source of CD8+ T cell epitopes in the boosting composition is a vaccinia virus vector such as MVA or NYVAC. Most preferred is the vaccinia strain modified virus ankara (MVA) or a strain
20 derived therefrom. Alternatives to vaccinia vectors include avipox vectors such as fowlpox or canarypox vectors. Particularly suitable as an avipox vector is a strain of canarypox known as ALVAC (commercially available as Kanapox), and strains derived therefrom.

Poxvirus genomes can carry a large amount of heterologous
25 genetic information. Other requirements for viral vectors for use in vaccines include good immunogenicity and safety. MVA is a replication-impaired vaccinia strain with a good safety record. In most cell types and normal human tissues, MVA does not replicate; limited replication of MVA is observed in a few transformed cell types such as BHK21 cells. It has
30 now been shown, by the results described herein, that recombinant MVA

and other non-replicating or replication-impaired strains are surprisingly and significantly better than conventional recombinant vaccinia vectors at generating a protective CD8+ T cell response, when administered in a boosting composition following priming with a DNA plasmid, a recombinant Ty-VLP or a recombinant adenovirus.

It will be evident that vaccinia virus strains derived from MVA, or independently developed strains having the features of MVA which make MVA particularly suitable for use in a vaccine, will also be suitable for use in the invention.

MVA containing an inserted string of epitopes (MVA-HM, which is described in the Examples) has been deposited at the European Collection of Animal Cell Cultures, CAMR, Salisbury, Wiltshire SP4 0JG, UK under accession no. V97060511 on 5 June 1997.

The term "non-replicating" or "replication-impaired" as used herein means not capable of replication to any significant extent in the majority of normal mammalian cells or normal human cells. Viruses which are non-replicating or replication-impaired may have become so naturally (i.e. they may be isolated as such from nature) or artificially e.g. by breeding *in vitro* or by genetic manipulation, for example deletion of a gene which is critical for replication. There will generally be one or a few cell types in which the viruses can be grown, such as CEF cells for MVA.

Replication of a virus is generally measured in two ways:

1) DNA synthesis and 2) viral titre. More precisely, the term "non-replicating or replication-impaired" as used herein and as it applies to poxviruses means viruses which satisfy either or both of the following criteria:

- 1) exhibit a 1 log (10 fold) reduction in DNA synthesis compared to the Copenhagen strain of vaccinia virus in MRC-5 cells (a human cell line);

- 2) exhibit a 2 log reduction in viral titre in HELA cells (a human cell line) compared to the Copenhagen strain of vaccinia virus.

Examples of poxviruses which fall within this definition are
5 MVA, NYVAC and avipox viruses, while a virus which falls outside the definition is the attenuated vaccinia strain M7.

Alternative preferred viral vectors for use in the priming composition according to the invention include a variety of different viruses, genetically disabled so as to be non-replicating or replication-impaired.
10 Such viruses include for example non-replicating adenoviruses such as E1 deletion mutants. Genetic disabling of viruses to produce non-replicating or replication-impaired vectors has been widely described in the literature (e.g. McLean *et al.* 1994).

Other suitable viral vectors for use in the priming composition
15 are vectors based on herpes virus and Venezuelan equine encephalitis virus (VEE) (Davies *et al.* 1996). Suitable bacterial vectors for priming include recombinant BCG and recombinant *Salmonella* and *Salmonella* transformed with plasmid DNA (Darji A *et al.* 1997 Cell 91: 765-775).

Alternative suitable non-viral vectors for use in the priming
20 composition include lipid-tailed peptides known as lipopeptides, peptides fused to carrier proteins such as KLH either as fusion proteins or by chemical linkage, whole antigens with adjuvant, and other similar systems. Adjuvants such as QS21 or SBAS2 (Stoute J A *et al.* 1997 N Engl J Medicine 226: 86-91) may be used with proteins, peptides or nucleic acids
25 to enhance the induction of T cell responses. These systems are sometimes referred to as "immunogens" rather than "vectors", but they are vectors herein in the sense that they carry the relevant CD8+ T cell epitopes.

There is no reason why the priming and boosting
30 compositions should not be identical in that they may both contain the

priming source of CD8+ T cell epitopes as defined in (i) above and the boosting source of CD8+ T cell epitopes as defined in (ii) above. A single formulation which can be used as a primer and as a booster will simplify administration. The important thing is that the primer contains at least the
5 priming source of epitopes as defined in (i) above and the booster contains at least the boosting source of epitopes as defined in (ii) above.

The CD8+ T cell epitopes either present in, or encoded by the priming and boosting compositions, may be provided in a variety of different forms, such as a recombinant string of one or two or more
10 epitopes, or in the context of the native target antigen, or a combination of both of these. CD8+ T cell epitopes have been identified and can be found in the literature, for many different diseases. It is possible to design epitope strings to generate a CD8+ T cell response against any chosen antigen that contains such epitopes. Advantageously, the epitopes in a
15 string of multiple epitopes are linked together without intervening sequences so that unnecessary nucleic acid and/or amino acid material is avoided. In addition to the CD8+ T cell epitopes, it may be preferable to include one or more epitopes recognised by T helper cells, to augment the immune response generated by the epitope string. Particularly suitable T
20 helper cell epitopes are ones which are active in individuals of different HLA types, for example T helper epitopes from tetanus (against which most individuals will already be primed). A useful combination of three T helper epitopes is employed in the examples described herein. It may also be useful to include B cell epitopes for stimulating B cell responses and
25 antibody production.

The priming and boosting compositions described may advantageously comprise an adjuvant. In particular, a priming composition comprising a DNA plasmid vector may also comprise granulocyte macrophage-colony stimulating factor (GM-CSF), or a plasmid

encoding it, to act as an adjuvant; beneficial effects are seen using GM-CSF in polypeptide form.

The compositions described herein may be employed as therapeutic or prophylactic vaccines. Whether prophylactic or therapeutic immunisation is the more appropriate will usually depend upon the nature of the disease. For example, it is anticipated that cancer will be immunised against therapeutically rather than before it has been diagnosed, while anti-malaria vaccines will preferably, though not necessarily be used as a prophylactic.

The compositions according to the invention may be administered via a variety of different routes. Certain routes may be favoured for certain compositions, as resulting in the generation of a more effective response, or as being less likely to induce side effects, or as being easier for administration. The present invention has been shown to be effective with gene gun delivery, either on gold beads or as a powder.

In further aspects, the invention provides:

- a method for generating a protective CD8+ T cell immune response against a pathogen or tumour, which method comprises administering at least one dose of a recombinant DNA plasmid encoding at least one CD8+ T cell epitope or antigen of the pathogen or cancer, followed by at least one dose of a non-replicating or replication-impaired recombinant pox virus encoding the same epitope or antigen;
- a method for generating a protective CD8+ T cell immune response against a pathogen or tumour, which method comprises administering at least one dose of a recombinant protein or particle comprising at least one epitope or antigen of the pathogen or cancer, followed by at least one dose of a recombinant MVA vector encoding the same epitope or antigen;

- the use of a recombinant non-replicating or replication-impaired pox virus vector in the manufacture of a medicament for boosting a CD8+ T cell immune response;
- the use of an MVA vector in the manufacture of a
5 medicament for boosting a CD8+ T cell immune response;
- a medicament for boosting a primed CD8+ T cell response against at least one target antigen or epitope, comprising a source of one or more CD8+ T cell epitopes of the target antigen, wherein the source of CD8+ T cell epitopes is a non-replicating or a replication-impaired
10 recombinant poxvirus vector, together with a pharmaceutically acceptable carrier; and
- the priming and/or boosting compositions described herein, in particulate form suitable for delivery by a gene gun; and methods of immunisation comprising delivering the compositions by means of a gene
15 gun.

Also provided by the invention are: the epitope strings described herein, including epitope strings comprising the amino acid sequences listed in table 1 and table 2; recombinant DNA plasmids encoding the epitope strings; recombinant Ty-VLPs comprising the epitope
20 strings; a recombinant DNA plasmid or non-replicating or replication impaired recombinant pox virus encoding the *P. falciparum* antigen TRAP; and a recombinant polypeptide comprising a whole or substantially whole protein antigen such as TRAP and a string of two or more epitopes in sequence such as CTL epitopes from malaria.

25

Example Formulations and Immunisation Protocols

Formulation 1

Priming Composition: DNA plasmid 1 mg/ml in PBS

Boosting Composition: Recombinant MVA, 10⁸ ffu in PBS

30

Protocol: Administer two doses of 1 mg of priming composition, i.m., at 0 and 3 weeks followed by two doses of booster intradermally at 6 and 9 weeks.

5 **Formulation 2**

Priming Composition: Ty-VLP 500 μ g in PBS

Boosting Composition: MVA, 10⁸ ffu in PBS

Protocol: Administer two doses of priming composition, i.m., at 0 and 3
10 weeks, then 2 doses of booster at 6 and 9 weeks. For tumour treatment, MVA is given i.v. as one of most effective routes.

Formulation 3

Priming Composition: Protein 500 μ g + adjuvant (QS-21)

15 Boosting Composition: Recombinant MVA, 10⁸ ffu in PBS

Protocol: Administer two doses of priming composition at 0 and 3 weeks and 2 doses of booster i.d. at 6 and 9 weeks.

20 **Formulation 4**

Priming Composition: Adenovirus vector, 10⁹ pfu in PBS

Boosting Composition: Recombinant MVA, 10⁸ ffu in PBS

Protocol: Administer one or two doses of priming composition
25 intradermally at 0 and 3 weeks and two doses of booster i.d. at 6 and 9 weeks.

The above doses and protocols may be varied to optimise protection.

Doses may be given between for example, 1 to 8 weeks apart rather than
30 2 weeks apart.

The invention will now be further described in the examples which follow.

EXAMPLES

5 EXAMPLE 1

Materials and Methods

Generation of the epitope strings.

The malaria epitope string was made up of a series of cassettes each encoding three epitopes as shown in Table 1, with
 10 restriction enzyme sites at each end of the cassette. Each cassette was constructed from four synthetic oligonucleotides which were annealed together, ligated into a cloning vector and then sequenced to check that no errors had been introduced. Individual cassettes were then joined together as required. The BamHI site at the 3' end of cassette C was fused to the
 15 BglII site at the 5' end of cassette A, destroying both restriction enzyme sites and encoding a two amino acid spacer (GS) between the two cassettes. Cassettes B, D and H were then joined to the string in the same manner. A longer string containing CABDHFE was also constructed in the same way.

20

Table 1. CTL epitopes of the malaria (M) string

Cassette	Epitope	Amino acid Sequence	DNA sequence	Type	HLA restriction
A	Ls8	KPNDKSLY	AAGCCGAACGACAAGTCCTTGTAT	CTL	B35
	Cp26	KPKDELDY	AAACCTAAGGACGAATTGGACTAC	CTL	B35
	Ls6	KPIVQYDNF	AAGCCAATCGTTCAATACGACAACCTC	CTL	B53
B	Tr42/43	ASKNKEKALII	GCCCCAAGAACAAGGAAAAGGCTTTGATCAT C	CTL	B8
	Tr39	GIAGGLALL	GGTATCGCTGGTGGTTTGGCCTTGTTG	CTL	A2.1
	Cp6	MNPNDPNRN V	ATGAACCCTAATGACCCAAACAGAAACGTC	CTL	B7

C	St8	MINAYLDKL	ATGATCAACGCCTACTTGGACAAGTTG	CTL	A2.2
	Ls50	ISKYEDEI	ATCTCCAAGTACGAAGACGAAATC	CTL	B17
	Pb9	SYIPSAEKI	TCCTACATCCCATCTGCCGAAAAGATC	CTL	mouse H2-K ^d
D	Tr26	HLGNVKYLV	CACTTGGGTAACGTTAAGTACTTGGTT	CTL	A2.1
	Ls53	KSLYDEHI	AAGTCTTTGTACGATGAACACATC	CTL	B58
	Tr29	LLMDCSGSI	TTATTGATGGACTGTTCTGGTTCTATT	CTL	A2.2
E	NANP	NANPNANPN ANPNANP	AACGCTAATCCAAACGCAAATCCGAACGCCA ATCCTAACGCGAATCCC	B cell	
	TRAP AM	DEWSPCSV CGKGTRSRK RE	GACGAATGGTCTCCATGTTCTGTCACTTGTG GTAAGGGTACTCGCTCTAGAAAGAGAGAA	Heparin binding motif	
F	Cp39	YLNKIQNSL	TACTTGAACAAAATTCAAACCTTTTG	CTL	A2.1
	La72	MEKLKELEK	ATGGAAAAGTTGAAAGAATTGGAAAAG	CTL	B8
	ex23	ATSVLAGL	GCTACTTCTGTCTTGGCTGGTTTG	CTL	B58
H	CSP	DPNANPNVD PNANPNV	GACCCAAACGCTAACCCAAACGTTGACCCA AACGCCAACCCAAACGTC	T helper	Universal epitopes
	BCG	QVHFQPLPP AVVKL	CAAGTTCACTTCCAACCATTCGCTCCGGCCG TTGTCAAGTTG	T helper	
	TT	QFIKANSKFI GITE	CAATTCATCAAGGCCAACTCTAAGTTCATCG GTATCACCGAA	T helper	

Table 1 Sequences included in the malaria epitope string. Each cassette consists of the epitopes shown above, in the order shown, with no additional sequence between epitopes within a cassette. A BglII site was added at the 5' end and a BamHI site at the 3' end, such that between cassettes in an epitope string the BamHI/BglII junction encodes GS. All epitopes are from *P. falciparum* antigens except for pb9 (*P. berghei*), BCG (*M. tuberculosis*) and TT (Tetanus). The amino acid and DNA sequences shown in the table have SEQ ID NOS. 1 to 40 in the order in which they appear.

Figure 1 shows the construct used to express Ty-VLP with the malaria epitope cassette CABDHFE. CTL epitopes are from *P. falciparum* STARP (sporozoite threonine- and asparagine-rich protein) (st), LSA-1 (liver stage antigen 1) (1s), CSP (circumsporozoite protein) (cp),

TRAP (thrombospondin-related adhesive protein) (tr), LSA-3 (liver stage antigen 3) (la) and Exp-1 (exported protein 1) (ex). Helper epitopes are from the *P. falciparum* CS protein, the *M. tuberculosis* 38Kd antigen and Tetanus Toxoid. NANP is the antibody epitope from CS and AM is the
 5 adhesion motif from *P. falciparum* TRAP (Muller *et al* 1993). The length of the complete string is 229 amino acids as shown in the table 1 legend, with the amino acid sequence:-

MINAYLDKLISKYEDEISYIPSAEKIGSKPNDKSLYKPKDEL DYKPIVQYDN
 FGSASKNKEKALIIGIAGGLALLMNPNDPNRNVGSHLGNVKYL VKSLYDE
 10 HILLMDCSGSIGSDPNANPNVDPNANPNVQVHFQPLPPAVVKLQFIKANS
 KFIGITEGSYLNKIQNSLMEKLKELEKATSVLAGLGSNANPNANPNANPNA
 NPDEWSPCSVTGCGKGRSRKREGSGK [SEQ ID NO: 41].

The HIV epitope string was also synthesised by annealing oligonucleotides. Finally the HIV and malaria epitope strings were fused
 15 by joining the *Bam*HI site at the 3' end of the HIV epitopes to the *Bgl*II site at the 5' end of cassettes CAB to form the HM string (Table 2)

Table 2 CTL epitopes of the HIV/SIV epitope string

Epitope	Restriction	Origin
YLKDQQLL	A24, B8	HIV-1 gp41
ERYLKDQQL	B14	HIV-1 gp41
EITPIGLAP	Mamu-B*01	SIV env
PPIPVGEIY	B35	HIV-1 p24
GEIYKRWII	B8	HIV-1 p24
KRWIILGLNK	B*2705	HIV-1 p24
IILGLNKIVR	A33	HIV-1 p24
LGLNKIVRMY	Bw62	HIV-1 p24
YNLTMKCR	Mamu-A*02	SIV env
RGPGRAFVTI	A2, H-2Dd	HIV-1 gp120
GRAFVTIGK	B*2705	HIV-1 gp120
TPYDINQML	B53	HIV-2 gag
CTPYDINQM	Mamu-A*01	SIV gag

RPQVPLRPMTY	B51	HIV-1 nef
QVPLRPMTYK	A*0301, A11	HIV-1 nef
VPLRPMTY	B35	HIV-1 nef
AVDLSHFLK	A11	HIV-1 nef
DLSHFLKEK	A*0301	HIV-1 nef
FLKEKGGL	B8	HIV-1 nef
ILKEPVHGV	A*0201	HIV-1 pol
ILKEPVHGVY	Bw62	HIV-1 pol
HPDIVIQY	B35	HIV-1 pol
VIYQYMDDL	A*0201	HIV-1 pol

Table 2 Sequences of epitopes from HIV or SIV contained in the H
 5 epitope string and assembled as shown in figure 2. The amino acids in the
 table have SEQ ID NOS: 42 to 64 in the order in which they appear.

Figure 2 shows a schematic outline of the H, M and HM
 proteins. The bar patterns on the schematic representations of the
 polypeptide proteins indicate the origin of the sequences (see tables 1 and
 10 2). The positions of individual epitopes and their MHC restrictions are
 depicted above and below the proteins. Pb is the only epitope derived from
 the protein of *P. berghei*. All other epitopes in the M protein originate from
 proteins of *P. falciparum*: cs – circumsporozoite protein, st – STARP, ls –
 LSA-1 and tr – TRAP. BCG – 38 kDa protein of *M. tuberculosis*; TT –
 15 tetanus toxin.

For the anti-tumour vaccine an epitope string containing CTL
 epitopes was generated, similar to the malaria and HIV epitope string. In
 this tumour epitope string published murine CTL epitopes were fused
 together to create the tumour epitope string with the amino acid sequence:
 20 **MLPYLGWLVF-AQHPNAELL-KHYLFRNL-SPSYVYHQF-IPNPLLGLD**
 [SEQ ID NO: 65]. CTL epitopes shown here were fused together. The first
 amino acid methionine was introduced to initiate translation.

Ty virus-like particles (VLPs).

The epitope string containing cassette CABDH was introduced into a yeast expression vector to make a C-terminal in-frame fusion to the TyA protein. When TyA or TyA fusion proteins are expressed in yeast from this vector, the protein spontaneously forms virus like particles which can be purified from the cytoplasm of the yeast by sucrose gradient centrifugation. Recombinant Ty-VLPs were prepared in this manner and dialysed against PBS to remove the sucrose before injection (c.f. Layton *et al.* 1996).

Adenoviruses

Replication-defective recombinant Adenovirus with a deletion of the E1 genes was used in this study (McGrory *et al.* 1988). The Adenovirus expressed *E. coli* β -galactosidase under the control of a CMV IE promoter. For immunisations, 10^7 pfu of virus were administered intradermally into the ear lobe.

Peptides

Peptides were purchased from Research Genetics (USA), dissolved at 10 mg/ml in DMSO (Sigma) and further diluted in PBS to 1 mg/ml. Peptides comprising CTL epitopes that were used in the experiments described herein are listed in table 3

Table 3 Sequence of CTL peptide epitopes

sequence	Antigen	MHC restriction
LPYLGWLVF	P1A tumour antigen	L ^d
SYIPSAEKI	<i>P. berghei</i> CSP	K ^d
RGPGRAFVTI	HIV gag	D ^d

TPHPARIGL	<i>E. coli</i> β -galactosidase	L ^d
TYQRTRALV	Influenza A virus NP	K ^d
SDYEGRLI	Influenza A virus NP	K ^k
ASNENMETM	Influenza A virus NP	D ^b
INVAFNRFL	<i>P. falciparum</i> TRAP	K ^b

The amino acid sequences in Table 3 have SEQ ID NOS: 66 to 73, in the order in which they appear in the Table.

5 *Plasmid DNA constructs*

A number of different vectors were used for constructing DNA vaccines. Plasmid pTH contains the CMV IE promoter with intron A, followed by a polylinker to allow the introduction of antigen coding sequences and the bovine growth hormone transcription termination sequence. The plasmid carries the ampicillin resistance gene and is capable of replication in *E. coli* but not mammalian cells. This was used to make DNA vaccines expressing each of the following antigens: *P. berghei* TRAP, *P. berghei* CS, *P. falciparum* TRAP, *P. falciparum* LSA-1 (278 amino acids of the C terminus only), the epitope string containing cassettes CABDH and the HM epitope string (HIV epitopes followed by cassettes CAB). Plasmid pSG2 is similar to pTH except for the antibiotic resistance gene. In pSG2 the ampicillin resistance gene of pTH has been replaced by a kanamycin resistance gene. pSG2 was used to make DNA vaccines expressing the following antigens: *P. berghei* PbCSP, a mouse tumour epitope string, the epitope string containing cassettes CABDH and the HM epitope string. Plasmid V1J-NP expresses influenza nucleoprotein under the control of a CMV IE promoter. Plasmids CMV-TRAP and CMV-LSA-1 are similar to pTH.TRAP and pTH. LSA-1 but do not contain intron A of the CMV promoter. Plasmids RSV.TRAP and RSV.LSA-1 contain the RSV promoter, SV40 transcription termination sequence and are tetracycline

resistant. For induction of β -galactosidase-specific CTL plasmid pcDNA3/His/LacZ (Invitrogen) was used. All DNA vaccines were prepared from *E. coli* strain DH5 α using Qiagen plasmid purification columns.

5 *Generation of recombinant vaccinia viruses*

Recombinant MVAs were made by first cloning the antigen sequence into a shuttle vector with a viral promoter such as the plasmid pSC11 (Chakrabarti *et al.* 1985; Morrison *et al.* 1989). *P. berghei* CS and *P. falciparum* TRAP, influenza nucleoprotein and the HM and mouse
10 tumour epitope polyepitope string were each expressed using the P7.5 promoter (Mackett *et al.* 1984), and *P. berghei* TRAP was expressed using the strong synthetic promoter (SSP; Carroll *et al.* 1995). The shuttle vectors, pSC11 or pMCO3 were then used to transform cells infected with wild-type MVA so that viral sequences flanking the promoter, antigen
15 coding sequence and marker gene could recombine with the MVA and produce recombinants. Recombinant viruses express the marker gene (β glucuronidase or β galactosidase) allowing identification of plaques containing recombinant virus. Recombinants were repeatedly plaque purified before use in immunisations. The recombinant NYVAC-PbCSP
20 vaccinia was previously described (Lanar *et al.* 1996). The wild type or Western Reserve (WR) strain of recombinant vaccinia encoding PbCSP was described previously (Satchidanandam *et al.* 1991).

Cells and culture medium

25 Murine cells and Epstein-Barr virus transformed chimpanzee and macaque B cells (BCL) were cultured in RPMI supplemented with 10% heat inactivated fetal calf serum (FCS). Splenocytes were restimulated with the peptides indicated (final concentration 1 μ g/ml) in MEM medium with 10% FCS, 2mM glutamine, 50U/ml penicillin, 50 μ M 2-
30 mercaptoethanol and 10mM Hepes pH7.2 (Gibco, UK).

Animals

Mice of the strains indicated, 6-8 weeks old were purchased from Harlan Olac (Shaws Farm, Blackthorn, UK). Chimpanzees H1 and
5 H2 were studied at the Biomedical Primate Research Centre at Rijswijk, The Netherlands. Macaques were studied at the University of Oxford.

Immunisations

Plasmid DNA immunisations of mice were performed by
10 intramuscular immunisation of the DNA into the musculus tibialis under anaesthesia. Mouse muscle was sometimes pre-treated with 50 µl of 1mM cardiotoxin (Latoxan, France) 5-9 days prior to immunisation as described by Davis *et al* (1993), but the presence or absence of such pre-treatment was not found to have any significant effect on immunogenicity or
15 protective efficacy. MVA immunisation of mice was performed by either intramuscular (i.m.), intravenous (into the lateral tail vein) (i.v.), intradermal (i.d.), intraperitoneal (i.p.) or subcutaneous (s.c.) immunisation. Plasmid DNA and MVA immunisation of the chimpanzees H1 and H2 was performed under anaesthesia by intramuscular immunisation of leg
20 muscles. For these chimpanzee immunisations the plasmid DNA was co-administered with 15 micrograms of human GM-CSF as an adjuvant. Recombinant MVA administration to the chimpanzees was by intramuscular immunisation under veterinary supervision. Recombinant human GM-CSF was purchased from Sandoz (Camberley, UK). For
25 plasmid DNA immunisations using a gene gun, DNA was precipitated onto gold particles. For intradermal delivery, two different types of gene guns were used, the Acell and the Oxford Bioscience device (PowderJect Pharmaceuticals, Oxford, UK).

ELISPOT assays

CD8+ T cells were quantified in the spleens of immunised mice without *in vitro* restimulation using the peptide epitopes indicated and the ELISPOT assay as described by Miyahara *et al* (1993). Briefly, 96-well nitrocellulose plates (Miliscreen MAHA, Millipore, Bedford UK) were coated with 15 µg/ml of the anti-mouse interferon-γ monoclonal antibody R4 (EACC) in 50 µl of phosphate-buffered saline (PBS). After overnight incubation at 4°C the wells were washed once with PBS and blocked for 1 hour at room temperature with 100 µl RPMI with 10% FCS. Splenocytes from immunised mice were resuspended to 1 x 10⁷ cells/ml and placed in duplicate in the antibody coated wells and serially diluted. Peptide was added to each well to a final concentration of 1 µg/ml. Additional wells without peptide were used as a control for peptide-dependence of interferon-γ secretion. After incubation at 37°C in 5%CO₂ for 12-18 hours the plates were washed 6 times with PBS and water. The wells were then incubated for 3 hours at room temperature with a solution of 1 µg/ml of biotinylated anti-mouse interferon-γ monoclonal antibody XMG1.2 (Pharmingen, CA, USA) in PBS. After further washes with PBS, 50 µl of a 1 µg/ml solution of streptavidin-alkaline-phosphatase polymer (Sigma) was added for 2 hours at room temperature. The spots were developed by adding 50 µl of an alkaline phosphatase conjugate substrate solution (Biorad, Hercules, CA, USA). After the appearance of spots the reaction was stopped by washing with water. The number of spots was determined with the aid of a stereomicroscope.

25

ELISPOT assays on the chimpanzee peripheral blood lymphocytes were performed using a very similar method employing the assay and reagents developed to detect human CD8 T cells (Mabtech, Stockholm).

30

CTL assays

CTL assays were performed using chromium labelled target cells as indicated and cultured mouse spleen cells as effector cells as described by Allsopp *et al.* (1996). CTL assays using chimpanzee or
5 macaque cells were performed as described for the detection of human CTL by Hill *et al.* (1992) using EBV-transformed autologous chimpanzee chimpanzee or macaque B cell lines as target cells.

P. berghei challenge

10 Mice were challenged with 2000 (BALB/c) or 200 (C57BL/6) sporozoites of the *P. berghei* ANKA strain in 200 µl RPMI by intravenous inoculation as described (Lanar *et al.* 1996). These sporozoites were dissected from the salivary glands of *Anopheles stephensi* mosquitoes maintained at 18°C for 20-25 days after feeding on infected mice. Blood-
15 stage malaria infection, indicating a failure of the immunisation, was detected by observing the appearance of ring forms of *P. berghei* in Giemsa-stained blood smears taken at 5-12 days post-challenge.

P. falciparum challenge

20 The chimpanzees were challenged with 20,000 *P. falciparum* sporozoites of the NF54 strain dissected from the salivary glands of *Anopheles gambiae* mosquitoes, by intravenous inoculation under anaesthesia. Blood samples from these chimpanzees were examined daily from day 5 after challenge by microscopy and parasite culture, in
25 order to detect the appearance of low levels of *P. falciparum* parasites in the peripheral blood.

P815 tumour challenges

Mice were challenged with 1×10^5 P815 cells in 200 µl of
30 PBS by intravenous inoculation. Animals were monitored for survival.

Influenza virus challenges

Mice were challenged with 100 haemagglutinating units (HA) of influenza virus A/PR/8/34 by intranasal inoculation. Following challenge
5 the animals were weighed daily and monitored for survival.

Determining peptide specific CTL using tetramers

Tetrameric complexes consisting of Mamu-A*01-heavy chain and β_2 -microglobulin were made as described by Ogg *et al* (1998). DNA
10 coding for the leaderless extracellular portion of the Mamu-A*01 MHC class I heavy chain was PCR-amplified from cDNA using 5'primer MamuNdeI: 5'-CCT GAC TCA GAC CAT ATG GGC TCT CAC TCC ATG [SEQ ID NO: 74] and 3' primer: 5'-GTG ATA AGC TTA ACG ATG ATT CCA CAC CAT TTT CTG TGC ATC CAG AAT ATG ATG CAG GGA TCC
15 CTC CCA TCT CAG GGT GAG GGG C [SEQ ID NO: 75]. The former primer contained a NdeI restriction site, the latter included a HindIII site and encoded for the biotinylation enzyme BirA substrate peptide. PCR products were digested with NdeI and HindIII and ligated into the same sites of the polylinker of bacterial expression vector pGMT7. The rhesus
20 monkey gene encoding a leaderless β_2 -microglobulin was PCR amplified from a cDNA clone using primers B2MBACK: 5'-TCA GAC CAT ATG TCT CGC TCC GTG GCC [SEQ ID NO: 76] and B2MFOR: 5'-TCA GAC AAG CTT TTA CAT GTC TCG ATC CCA C [SEQ ID NO: 77] and likewise cloned into the NdeI and HindIII sites of pGMT7. Both chains were
25 expressed in *E. coli* strain BL-21, purified from inclusion bodies, refolded in the presence of peptide CTPYDINQM [SEQ ID NO: 54], biotinylated using the BirA enzyme (Avidity) and purified with FPLC and monoQ ion exchange columns. The amount of biotinylated refolded MHC-peptide
30 complexes was estimated in an ELISA assay, whereby monomeric complexes were first captured by conformation sensitive monoclonal

antibody W6/32 and detected by alkaline phosphatase (AP) –conjugated streptavidin (Sigma) followed by colorimetric substrate for AP. The formation of tetrameric complexes was induced by addition of phycoerythrin (PE)-conjugated streptavidin (ExtrAvidin; Sigma) to the refolded biotinylated monomers at a molar ratio of MHC-peptide : PE-streptavidin of 4 : 1. The complexes were stored in the dark at 4°C. These tetramers were used to analyse the frequency of Mamu-A*01/gag-specific CD8+ T cells in peripheral blood lymphocytes (PBL) of immunised macaques.

10

EXAMPLE 2

Immunogenicity Studies in Mice

Previous studies of the induction of CTL against epitopes in the circumsporozoite (CS) protein of *Plasmodium berghei* and *Plasmodium yoelii* have shown variable levels of CTL induction with different delivery systems. Partial protection has been reported with plasmid DNA (Sedegah *et al.* 1994), influenza virus boosted by replicating vaccinia virus (Li *et al.* 1991), adenovirus (Rodrigues *et al.* 1997) and particle delivery systems (Schodel *et al.* 1994). Immunisation of mice intramuscularly with 50 micrograms of a plasmid encoding the CS protein produced moderate levels of CD8+ cells and CTL activity in the spleens of these mice after a single injection (Figures 3, 4).

For comparison groups of BALB/c mice (n = 5) were injected intravenously with 10⁶ ffu/pfu of recombinant vaccinia viruses of different strains (WR, NYVAC and MVA) all expressing *P. berghei* CSP. The frequencies of peptide-specific CD8+ T cells were measured 10 days later in an ELISPOT assay. MVA.PbCSP induced 181 +/- 48, NYVAC 221 +/- 27 and WR 94 +/- 19 (mean +/- standard deviation) peptide-specific CD8+ T cells per million splenocytes. These results show that surprisingly replication-impaired vaccinia viruses are superior to replicating strains in

30

priming a CD8+ T cell response. We then attempted to boost these moderate CD8+ T cell responses induced by priming with either plasmid DNA or MVA using homologous or heterologous vectors. A low level of CD8+ T cells was observed after two immunisations with CS recombinant DNA vaccine alone, the recombinant MVA vaccine alone or the recombinant MVA followed by recombinant DNA (Figure 3). A very much higher level of CD8+ T cells was observed by boosting the DNA-primed immune response with recombinant MVA. In a second experiment using ten mice per group the enhanced immunogenicity of the DNA/MVA sequence was confirmed: DNA/MVA 856 +/- 201; MVA/DNA 168 +/- 72; MVA/MVA 345 +/- 90; DNA/DNA 92 +/- 46. Therefore the sequence of a first immunisation with a recombinant plasmid encoding the CS protein followed by a second immunisation with the recombinant MVA virus yielded the highest levels of CD8+ T lymphocyte response after immunisation.

Figure 3 shows malaria CD8 T cell ELISPOT data following different immunisation regimes. Results are shown as the number of peptide-specific T cells per million splenocytes. Mice were immunised either with the PbCSP-plasmid DNA or the PbCSP-MVA virus or combinations of the two as shown on the X axis, at two week intervals and the number of splenocytes specific for the pb9 malaria epitope assayed two weeks after the last immunisation. Each point represents the number of spot-forming cells (SFCs) measured in an individual mouse. The highest level of CD8+ T cells was induced by priming with the plasmid DNA and boosting with the recombinant MVA virus. This was more immunogenic than the reverse order of immunisation (MVA/DNA), two DNA immunisations (DNA/DNA) or two MVA immunisations (MVA/MVA). It was also more immunogenic than the DNA and MVA immunisations given simultaneously (DNA + MVA 2w), than one DNA immunisation (DNA 4w) or one MVA immunisation given at the earlier or later time point (MVA 2w and MVA 4w).

Figure 4 shows that malaria CD8 T cell ELISPOT and CTL levels are substantially boosted by a recombinant MVA immunisation following priming with a plasmid DNA encoding the same antigen. **A and C.** CD8+ T cell responses were measured in BALB/c mice using the γ -interferon ELISPOT assay on fresh splenocytes incubated for 18 h with the
5 interferon ELISPOT assay on fresh splenocytes incubated for 18 h with the K^d restricted peptide SYIPSAEKI [SEQ ID NO: 67] from *P. berghei* CSP and the L^d restricted peptide TPHPARIGL [SEQ ID NO: 69] from *E. coli* β -galactosidase. Note that the ELISPOT counts are presented on a logarithmic scale. **B and D.** Splenocytes from the same mice were also
10 assayed in conventional ^{51}Cr -release assays at an effector: target ration of 100:1 after 6 days of *in vitro* restimulation with the same peptides (1 $\mu\text{g/ml}$).

The mice were immunised with plasmid DNA expressing either *P. berghei* CSP and TRAP, *PbCSP* alone, the malaria epitope
15 cassette including the *P. berghei* CTL epitope (labelled pTH.M), or β -galactosidase. ELISPOT and CTL levels measured in mice 23 days after one DNA immunisation are shown in A and B respectively. The same assays were performed with animals that received additionally 1×10^7 ffu of recombinant MVA expressing the same antigen(s) two weeks after the
20 primary immunisation. The ELISPOT and CTL levels in these animals are shown in C and D respectively. Each bar represents data from an individual animal.

Studies were also undertaken of the immunogenicity of the epitope string HM comprising both HIV and malaria epitopes in tandem.
25 Using this epitope string again the highest levels of CD8+ T cells and CTL were generated in the spleen when using an immunisation with DNA vaccine followed by an immunisation with a recombinant MVA vaccine (Table 4, Figure 5).

Table 4 Immunogenicity of various DNA/MVA combinations as determined by ELISPOT assays

	Immunisation 1	Immunisation 2	HIV epitope	Malaria epitope
5	DNA-HM	DNA-HM	56 ± 26	4 ± 4
	MVA-HM	MVA-HM	786 ± 334	238 ± 106
10	MVA-HM	DNA-HM	306 ± 78	58 ± 18
	DNA-HM	MVA-HM	1000 ± 487	748 ± 446
	None	DNA-HM	70 ± 60	100 ± 10
15	None	MVA-HM	422 ± 128	212 ± 94

Table 4 shows the results of ELISPOT assays performed to measure the levels of specific CD8⁺ T cells to HIV and malaria epitopes following different immunisation regimes of plasmid DNA and MVA as indicated. The numbers are spot-forming cells per million splenocytes. The HM epitope string is illustrated in figure 2. BALB/c mice were used in all cases. The malaria epitope was pb9 as in figures 2 and 3. The HIV epitope was RGPGRFVFI [SEQ ID NO: 51]. The immunisation doses were 50 µg of plasmid DNA or 10⁷ focus-forming units (ffu) of recombinant MVA. All immunisations were intramuscular. The interval between immunisations 1 and 2 was from 14-21 days in all cases.

Figure 5 shows the CTL responses induced in BALB/c mice to malaria and HIV epitopes by various immunisation regimes employing plasmid DNA and recombinant MVA. Mice were immunised intramuscularly as described in the legend to table 3 and in methods. High levels of CTL (>30% specific lysis at effector/target ration of 25:1) were observed to both the malaria and HIV epitopes only after priming with

plasmid DNA and boosting with the recombinant MVA. The antigen used in this experiment is the HIV-malaria epitope string. The recombinant MVA is denoted MVA.HM and the plasmid DNA expressing this epitope string is denoted pTH.HM. Levels of specific lysis at various effector to target ratios are shown. These were determined after 5 days *in vitro* restimulation of splenocytes with the two peptides pb9 and RGPGRAFVTI [SEQ ID NO: 51].

Comparison of numerous delivery systems for the induction of CTL was reported by Allsopp *et al.* (1996). Recombinant Ty-virus like particles (Ty-VLPs) and lipid-tailed malaria peptides both gave good CTL induction but Ty-VLPs were better in that they required only a single immunising dose for good CTL induction. However, as shown here even two doses of Ty particles fail to induce significant protection against sporozoite challenge (Table 7, line 1). Immunisation with a recombinant modified vaccinia Ankara virus encoding the circumsporozoite protein of *P. berghei* also generates good levels of CTL. However, a much higher level of CD8+ T cell response is achieved by a first immunisation with the Ty-VLP followed by a second immunisation with the MVA CS vaccine (Table 5).

Table 5 Immunogenicity of various Ty-VLP/MVA combinations as determined by ELISPOT and CTL assays

Immunisation 1	Immunisation 2	ELISPOT No	%Specific Lysis
Ty-CABDH	Ty- CABDH	75	15
MVA.PbCSP	MVA.PbCSP	38	35
Ty-CABDH	MVA.PbCSP	225	42
Ty- CABDH	MVA.HM	1930	nd

Table 5 Results of ELISPOT and CTL assays performed to measure the levels of specific CD8+ T cells to the malaria epitope pb9 following different immunisation regimes of Ty-VLPs and recombinant MVA virus as indicated. The CTL and ELISPOT data are from different experiments.

- 5 The ELISPOT levels (spots per million splenocytes) are measured on un-restimulated cells and the CTL activity, indicated as specific lysis at an effector to target ratio of 40:1, on cells restimulated with pb9 peptide *in vitro* for 5-7 days. Both represent mean levels of three mice. BALB/c mice were used in all cases. The immunisation doses were 50 µg of Ty-VLP or
- 10 10⁷ ffu (foci forming units) of recombinant MVA. All immunisations were intramuscular. The interval between immunisations 1 and 2 was from 14-21 days. MVA.HM includes cassettes CAB.

Priming of an immune response with DNA delivered by a gene gun

15 ***and boosting with recombinant MVA***

Immunogenicity and challenge.

- The use of a gene gun to deliver plasmid DNA intradermally and thereby prime an immune response that could be boosted with recombinant MVA was investigated. Groups of BALB/c mice were
- 20 immunised with the following regimen:

- I) Three gene gun immunisations with pTH.PbCSP (4 mg per immunisation) at two week intervals
- II) Two gene gun immunisations followed by MVA i.v. two weeks later
- 25 III) One intramuscular DNA immunisation followed by MVA i.v. two weeks later.

The immunogenicity of the three immunisation regimens was analysed using ELISPOT assays. The highest frequency of specific T cells was observed with two gene gun immunisations followed by an MVA i.v.

boost and the intramuscular DNA injection followed an MVA i.v. boost (Figure 6).

Figure 6 shows the results of ELISPOT assays performed to measure the levels of specific CD8⁺ T cells to the malaria epitope pb9 following different immunisation regimes. Groups of BALB/c mice (n= 3) were immunised as indicated (g.g. = gene gun). The time between all immunisations was 14 days. ELISPOT assays were done two weeks after the last immunisation.

10 ***CTL induction to the same antigen in different mouse strains***

To address the question whether the boosting effect described above in BALB/c mice with two CTL epitopes SYIPSAEKI [SEQ ID NO: 67] derived from *P. berghei* CSP and RGPGRAFTI [SEQ ID NO: 68] derived from HIV) is a universal phenomenon, two sets of experiments were carried out. CTL responses to the influenza nucleoprotein were studied in five inbred mouse strains. In a first experiment three published murine CTL epitopes derived from the influenza nucleoprotein were studied (see Table 3). Mice of three different H-2 haplotypes, BALB/c and DBA/2 (H-2^d), C57BL/6 and 129 (H-2^b); CBA/J (H-2^k), were used. One set of animals was immunised twice at two week intervals with the plasmid V1J-NP encoding the influenza nucleoprotein. Another set of identical animals was primed with V1J-NP and two weeks later boosted intravenously with 10⁶ ffu of MVA.NP, expressing influenza virus NP. The levels of CTL in individual mice were determined in a ⁵¹Cr-release assay with peptide re-stimulated splenocytes. As shown in Figure 7, the DNA priming/MVA boosting immunisation regimen induced higher levels of lysis in all the mouse strains analysed and is superior to two DNA injections.

Figure 7 shows the CTL responses against influenza NP in different mouse strains. Mice of different strains were immunised twice two weeks apart with a DNA vaccine V1J-NP encoding for the influenza

nucleoprotein (open circles) or primed with the same DNA vaccine and two weeks later boosted with recombinant MVA expressing influenza virus nucleoprotein (closed circles). Two weeks after the last immunisation splenocytes were restimulated in vitro with the respective peptides (Table 3). The CTL activity was determined in a standard ^{51}Cr -release assay with MHC class I-matched target cells.

CTL induction to different antigens in different mouse strains

The effect of MVA boosting on plasmid DNA-primed immune responses was further investigated using different antigens and different inbred mouse strains. Mice of different strains were immunised with different antigens using two DNA immunisations and compared with DNA/MVA immunisations. The antigens used were *E. coli* β -galactosidase, the malaria/HIV epitope string, a murine tumour epitope string and *P. falciparum* TRAP. Compared with two DNA immunisations the DNA-priming/MVA-boosting regimen induced higher levels of CTL in all the different mouse strains and antigen combinations tested (Figure 8).

Figure 8 shows CTL responses against different antigens induced in different inbred mouse strains. Mice were immunised with two DNA vaccine immunisations two weeks apart (open circles) or primed with a DNA vaccine and two weeks later boosted with a recombinant MVA expressing the same antigen (closed circles). The strains and antigens were: C57BL/6; *P. falciparum* TRAP in A. DBA/2; *E. coli* β -galactosidase in B. BALB/c; HM epitope string CTL activity against malaria peptide (pb9) in C. DBA/2; HM epitope string CTL activity against pb9 in D. BALB/c; HM epitope string CTL activity against HIV peptide in E. DBA/2; HM epitope string CTL activity against HIV peptide in F. BALB/c; tumour epitope string CTL activity against P1A-derived peptide in G. DBA/2; tumour epitope string CTL activity against P1A-derived peptide in H. Sequences of peptide epitopes are shown in table 3. Each curve shows the data for an individual mouse.

Sporozoites can efficiently prime an immune response that is boostable by MVA

Humans living in malaria endemic areas are continuously exposed to sporozoite inoculations. Malaria-specific CTL are found in these naturally exposed individuals at low levels. To address the question whether low levels of sporozoite induced CTL responses can be boosted by MVA, BALB/c mice were immunised with irradiated (to prevent malaria infection) *P. berghei* sporozoites and boosted with MVA. Two weeks after the last immunisation splenocytes were re-stimulated and tested for lytic activity. Two injections with 50 or 300 + 500 sporozoites induced very low or undetectable levels of lysis. Boosting with MVA induced high levels of peptide specific CTL. MVA alone induced only moderate levels of lysis (Figure 9).

Figure 9 shows sporozoite-primed CTL responses are substantially boosted by MVA. Mice were immunised with two low doses (50 + 50) of irradiated sporozoites in A. two high doses (300 + 500) of sporozoites in B; mice were boosted with MVA.PbCSP following low-dose sporozoite priming in D; high dose sporozoite priming in E. CTL responses following immunisation with MVA.PbCSP are shown in C.

Recombinant adenoviruses as priming agent

The prime-boost immunisation regimen has been exemplified using plasmid DNA and recombinant Ty-VLP as priming agent. Here an example using non-replicating adenoviruses as the priming agent is provided. Replication-deficient recombinant Adenovirus expressing *E. coli* β -galactosidase (Adeno-GAL) was used. Groups of BALB/c mice were immunised with plasmid DNA followed by MVA or with Adenovirus followed by MVA. All antigen delivery systems used encoded *E. coli* β -galactosidase. Priming a CTL response with plasmid DNA or Adenovirus and boosting with MVA induces similar levels of CTL (Figure 10).

Figure 10 shows CTL responses primed by plasmid DNA or recombinant Adenovirus and boosted with MVA. Groups of BALB/c mice (n=3) were primed with plasmid DNA A or recombinant Adenovirus expressing β -galactosidase B. Plasmid DNA was administered intramuscularly, MVA intravenously and Adenovirus intradermally. Splenocytes were restimulated with peptide TPHPARIGL [SEQ ID NO: 69] two weeks after the last immunisation. CTL activity was tested with peptide-pulsed P815 cells.

Immunogenicity of the DNA prime vaccinia boost regimen depends on the replication competence of the strain of vaccinia virus used

The prime boosting strategy was tested using different strains of recombinant vaccinia viruses to determine whether the different strains with strains differing in their replication competence may differ in their ability to boost a DNA-primed CTL response. Boosting with replication-defective recombinant vaccinia viruses such as MVA and NYVAC resulted in the induction of stronger CTL responses compared to CTL responses following boosting with the same dose of replication competent WR vaccinia virus (Figure 11).

Figure 11 shows CTL responses in BALB/c mice primed with plasmid DNA followed by boosting with different recombinant vaccinia viruses. Animals were primed with pTH.PbCSP 50 μ g/mouse i.m. and two weeks later boosted with different strains of recombinant vaccinia viruses (10^6 pfu per mouse i.v.) expressing PbCSP. The different recombinant vaccinia virus strains were MVA in A; NYVAC in B and WR in C. The superiority of replication-impaired vaccinia strains over replicating strains was found in a further experiment. Groups of BALB/c mice (n = 6) were primed with 50 μ g/animal of pSG2.PbCSP (i.m.) and 10 days later boosted i.v. with 10^6 ffu/pfu of recombinant MVA, NYVAC and WR expressing PbCSP. The frequencies of peptide-specific CD8+ T cells were determined using the ELISPOT assay. The frequencies were: MVA 1103

+/- 438, NYVAC 826 +/- 249 and WR 468 +/- 135. Thus using both CTL assays and ELISPOT assays as a measure of CD8 T cell immunogenicity a surprising substantially greater immunogenicity of the replication-impaired vaccinia strains was observed compared to the replication competent strain.

The use of recombinant canary or fowl pox viruses for boosting CD8+ T cell responses

Recombinant canary pox virus (rCPV) or fowl pox virus (rFVP) are made using shuttle vectors described previously (Taylor *et al.* Virology 1992, 187: 321-328 and Taylor *et al.* Vaccine 1988, 6: 504-508). The strategy for these shuttle vectors is to insert the gene encoding the protein of interest preceded by a vaccinia-specific promoter between two flanking regions comprised of sequences derived from the CPV or FPV genome. These flanking sequences are chosen to avoid insertion into essential viral genes. Recombinant CPV or FPV are generated by *in vivo* recombination in permissive avian cell lines i.e. primary chicken embryo fibroblasts. Any protein sequence of antigens or epitope strings can be expressed using fowl pox or canary pox virus. Recombinant CPV or FPV is characterised for expression of the protein of interest using antigen-specific antibodies or including an antibody epitope into the recombinant gene. Recombinant viruses are grown on primary CEF. An immune response is primed using plasmid DNA as described in Materials and Methods. This plasmid DNA primed immune response is boosted using 10^7 ffu/pfu of rCPV or rFPV inoculated intravenously, intradermally or intramuscularly. CD8+ T cell responses are monitored and challenges are performed as described herein.

EXAMPLE 3

Malaria Challenge Studies in Mice

To assess the protective efficacy of the induced levels of CD8+ T cell response immunised BALB/c or C57BL/6 mice were challenged by intravenous injection with 2000 or 200 *P. berghei* sporozoites. This leads to infection of liver cells by the sporozoites. However, in the presence of a sufficiently strong T lymphocyte response against the intrahepatic parasite no viable parasite will leave the liver and no blood-stage parasites will be detectable. Blood films from challenged mice were therefore assessed for parasites by microscopy 5-12 days following challenge.

BALB/c mice immunised twice with a mixture of two plasmid DNAs encoding the CS protein and the TRAP antigen, respectively, of *P. berghei* were not protected against sporozoite challenge. Mice immunised twice with a mixture of recombinant MVA viruses encoding the same two antigens were not protected against sporozoite challenge. Mice immunised first with the two recombinant MVAs and secondly with the two recombinant plasmids were also not protected against sporozoite challenge. However, all 15 mice immunised first with the two plasmid DNAs and secondly with the two recombinant MVA viruses were completely resistant to sporozoite challenge (Table 6 A and B).

To assess whether the observed protection was due to an immune response to the CS antigen or to TRAP or to both, groups of mice were then immunised with each antigen separately (Table 6 B). All 10 mice immunised first with the CS plasmid DNA and secondly with the CS MVA virus were completely protected against sporozoite challenge. Fourteen out of 16 mice immunised first with the TRAP plasmid DNA vaccine and secondly with the TRAP MVA virus were protected against sporozoite challenge. Therefore the CS antigen alone is fully protective

when the above immunisation regime is employed and the TRAP antigen is substantially protective with the same regime.

The good correlation between the induced level of CD8+ T lymphocyte response and the degree of protection observed strongly suggests that the CD8+ response is responsible for the observed protection. In previous adoptive transfer experiments it has been demonstrated that CD8+ T lymphocyte clones against the major CD8+ T cell epitope in the *P. berghei* CS protein can protect against sporozoite challenge. To determine whether the induced protection was indeed mediated by CD8+ T cells to this epitope we then employed a plasmid DNA and a recombinant MVA encoding only this nine amino acid sequence from *P. berghei* as a part of a string of epitopes (Table 6 B). (All the other epitopes were from micro-organisms other than *P. berghei*). Immunisation of 10 mice first with a plasmid encoding such an epitope string and secondly with a recombinant MVA also encoding an epitope string with the *P. berghei* CTL epitope led to complete protection from sporozoite challenge (Table 6 B). Hence the induced protective immune response must be the CTL response that targets this nonamer peptide sequence.

Table 6 Results of mouse challenge experiments using different combinations of DNA and MVA vaccine

	Immunisation 1	Immunisation 2	No. Infected/ No. challenged	%Protection
5	A. Antigen used: PbCSP + PbTRAP			
	DNA	DNA	5/5	0%
	MVA	MVA	9/10	10%
	DNA	MVA	0/5	100%
10	MVA	DNA	5/5	0%
	Control mice immunised with β -galactosidase			
	DNA	DNA	5/5	0%
	MVA	MVA	5/5	0%
15	DNA	MVA	5/5	0%
	MVA	DNA	5/5	0%
	B.			
	DNA (CSP + TRAP)	MVA (CSP + TRAP)	0/10	100%
20	DNA (CSP)	MVA (CSP)	0/10	100%
	DNA (TRAP)	MVA (TRAP)	2/16	88%
	DNA (epitope)	MVA (epitope)	0/11	100%
	DNA (beta-gal)	MVA (beta-gal)	6/7	14%
	none	none	9/10	10%
25				

Table 6 Results of two challenge experiments (A and B) using different immunisation regimes of plasmid DNA and MVA as indicated. BALB/c mice were used in all cases. The immunisation doses were 50 μ g of plasmid DNA or 10^6 ffu of recombinant MVA. The interval between immunisations 1 and 2 was from 14-21 days in all cases. Challenges were performed at 18-29 days after the last immunisation by i.v. injection of 2000 *P. berghei* sporozoites and blood films assessed at 5, 8 and 10 days post challenge. CSP and TRAP indicate the entire *P. berghei* antigen and 'epitope' indicates the cassettes of epitopes shown in table 1 containing

only a single *P. berghei* K^d-restricted nonamer CTL epitope. Note that in experiment B immunisation with the epitope string alone yields 100% protection

Mice immunised twice with recombinant Ty-VLPs encoding pb9 were fully susceptible to infection. Similarly mice immunised twice with the recombinant MVA encoding the full CS protein were fully susceptible to infection. However, the mice immunised once with the Ty-VLP and subsequently once with the recombinant MVA showed an 85% reduction in malaria incidence when boosted with MVA expressing the full length CS protein, and 95% when MVA expressing the HM epitope string which includes pb9 was used to boost (Table 7).

Table 7 Results of challenge experiments using different immunisation regimes of Ty-VLPs and MVA

Immunisation 1	Immunisation 2	No. Infected/No.challenged	%Protection
Ty-CABDHFE	Ty- CABDHFE	7/8	13%
Ty-CABDH	MVA.PbCSP	2/13	85%
Ty- CABDHFE	MVA-NP	5/5	0%
MVA.PbCSP	MVA.PbCSP	6/6	0%
MVA.HM	Ty- CABDHFE	14/14	0%
Ty- CABDHFE	MVA.HM	1/21	95%
none	MVA.HM	8/8	0%
none	none	11/12	9%

Table 7 Results of challenge experiments using different immunisation regimes of Ty-VLPs and MVA as indicated. BALB/c mice were used in all cases. Immunisations were of 50 µg of Ty-VLP or 10⁷ ffu of recombinant MVA administered intravenously. The interval between immunisations 1 and 2 was from 14-21 days in all cases. Challenges were performed at 18-29 days after the last immunisation by i.v. injection of

2000 *P. berghei* sporozoites and blood films assessed at 5, 8 and 10 days post challenge. CSP indicates the entire *P. berghei* antigen. Ty-VLPs carried epitope cassettes CABDH or CABDHFE as described in table 1. MVA.HM includes cassettes CAB.

5 To determine whether the enhanced immunogenicity and protective efficacy observed by boosting with a recombinant MVA is unique to this particular vaccinia virus strain or is shared by other recombinant vaccinas the following experiment was performed. Mice were immunised with the DNA vaccine encoding *P. berghei* CS protein and boosted with
10 either (i) recombinant MVA encoding this antigen; (ii) recombinant wild-type vaccinia virus (Western Reserve strain) encoding the same antigen (Satchidanandam *et al.* 1991), or (iii) recombinant NYVAC (COPAK) virus (Lanar *et al.* 1996) encoding the same malaria antigen. The highest degree of protection was observed with boosting by the MVA recombinant,
15 80% (Table 8). A very low level of protection (10%) was observed by boosting with the wild-type recombinant vaccinia virus and a significant level of protection, 60%, by boosting with the NYVAC recombinant. Hence the prime-boost regime we describe induces protective efficacy with any non-replicating vaccinia virus strain. Both the MVA recombinant and
20 NYVAC were significantly ($P < 0.05$ for each) better than the WR strain recombinant.

Table 8 Challenge data results for DNA boosted with various vaccinia strain recombinants.

25

Immunisation 1	Immunisation 2	No. Infected/No. challenged	%Protection
DNA-beta gal.	MVA.NP	8/8	0%
DNA-CSP	MVA-CSP	2/10	80%
DNA-CSP	WR-CSP	9/10	10%
30 DNA-CSP	NYVAC-CSP	4/10	60%

Table 8 Results of a challenge experiment using different immunisation regimes of plasmid DNA and various vaccinia recombinants as indicated. BALB/c mice were used in all cases. The immunisation doses were 50 µg of plasmid DNA or 10⁶ ffu/pfu of recombinant MVA or 5 10⁴ ffu/pfu of recombinant wild type (WR) vaccinia or 10⁶ ffu/pfu of recombinant NYVAC. Because the WR strain will replicate in the host and the other strains will not, in this experiment a lower dose of WR was used. The interval between immunisations 1 and 2 was 23 days. Challenges were performed at 28 days after the last immunisation by i.v. injection of 10 2000 *P. berghei* sporozoites and blood films assessed at 7, 9 and 11 days post challenge. pbCSP indicates the entire *P. berghei* antigen and NP the nucleoprotein antigen of influenza virus (used as a control antigen). The first immunisation of group A mice was with the plasmid DNA vector expressing beta galactosidase but no malaria antigen.

15 In a further experiment shown in Table 8, mice were immunised with the DNA vaccine encoding *P. berghei* CS protein and boosted with either (i) recombinant MVA encoding this antigen; (ii) recombinant WR vaccinia virus encoding the same antigen or (iii) recombinant NYVAC (COPAK) virus encoding the same malaria antigen, 20 all at 10⁶ ffu/pfu. A high and statistically significant degree of protection was observed with boosting with recombinant NYVAC (80%) or recombinant MVA (66%). A low and non-significant level of protection (26%) was observed by boosting with the WR recombinant vaccinia virus (Table 9). MVA and NYVAC boosting each gave significantly more 25 protection than WR boosting ($P = 0.03$ and $P = 0.001$ respectively). These data re-emphasise that non-replicating pox virus strains are better boosting agents for inducing high levels of protection.

Table 9 Influence of different recombinant vaccinia strains on protection.

Immunisation 1 DNA	Immunisation 2	No. inf./ No. chall.	% protection
CSP	MVA.PbCSP	5/15	66
CSP	NYVAC.PbCSP	2/15	80
CSP	WR.PbCSP	11/15	26
β -galactosidase	MVA.NP	8/8	0

5

Table 9 Results of challenge experiments using different immunisation regimes of plasmid DNA and replication incompetent vaccinia recombinants as boosting immunisation. BALB/c mice were used in all cases. The immunisation doses were 50 μ g of plasmid DNA or 10⁶ ffu/pfu of recombinant MVA or recombinant wild type (WR) vaccinia or recombina

10 ffu/pfu of recombinant MVA or recombinant wild type (WR) vaccinia or recombina

NYVAC. The interval between immunisations 1 and 2 was 23 days. Challenges were performed at 28 days after the last immunisation by i.v. injection of 2000 *P. berghei* sporozoites and blood films assessed at 7, 9 and 11 days post challenge. PbCSP indicates the entire *P. berghei*

15 antigen and NP the nucleoprotein antigen of influenza virus (used as a control antigen). The control immunisation was with a plasmid DNA vector expressing β -galactosidase followed by MVA.NP.

Alternative routes for boosting immune responses with recombinant MVA

20

Intravenous injection of recombinant MVA is not a preferred route for immunising humans and not feasible in mass immunisations.

Therefore different routes of MVA boosting were tested for their immunogenicity and protective efficacy.

Mice were primed with plasmid DNA i.m. Two weeks later they were boosted with MVA administered via the following routes: intravenous (i.v.), subcutaneous (s.c.), intraperitoneal (i.p.), intramuscular (i.p.) and intradermal (i.d.). Two weeks after this boost peptide-specific CD8⁺ T cells were determined in an ELISPOT assay. The most effective route which induced the highest levels were i.v. and i.d inoculation of MVA. The other routes gave moderate to poor responses (Figure 12).

Figure 12 shows frequencies of peptide-specific CD8⁺ T cells following different routes of MVA boosting. Results are shown as the number of spot-forming cells (SFC) per one million splenocytes. Mice were primed with plasmid DNA and two weeks later boosted with MVA via the indicated routes. The number of splenocytes specific for the SYIPSAEKI [SEQ ID NO: 67] peptide was determined in INF- γ ELISPOT assays two weeks after the last immunisation. Each bar represents the mean number of SFCs from three mice assayed individually.

Boosting via the i.v. route was compared with the i.d. and i.m route in a challenge experiment. The i.d route gave high levels of protection (80% protection). In the group of animals that were boosted via the i.m. route, 50% of the animals were protected. Complete protection was achieved with MVA boost administered i.v. (Table 10)

Table 10 Influence of the route of MVA administration on protective efficacy

Immunisation 1 DNA	Immunisation 2 MVA	No. infected/ No. challenged	% protection
CSP	CSP i.v.	*0/20	100
CSP	CSP i.d	2/10	80
CSP	CSP i.m.	5/10	50

Epitope	epitope i.v.	1/10	90
NP	NP i.v.	10/10	0

* culminative data from two independent experiments

Table 10 Results from challenge experiments using different routes of MVA boosting immunisation. Animals were primed by intramuscular plasmid DNA injection and two weeks later boosted with the indicated recombinant MVA (10^6 ffu/mouse) administered via the routes indicated. The mice were challenged 16 days after the last immunisation with 2000 P. berghei sporozoites and screened for blood stage parasitemia at day 8 and 10 post challenge. Epitope indicates the polypeptide string HM.

10

Alternative routes of DNA priming: The use of a gene gun to prime peptide specific CD8+ T cells

Gene gun delivery is described in detail in for example in Eisenbraun *et al.* DNA Cell Biol. 1993, 12: 791-797 and Degano *et al.* Vaccine 1998, 16: 394-398.

The mouse malaria challenge experiments described so far using plasmid DNA to prime an immune response used intramuscular injection of plasmid DNA. Intradermal delivery of plasmid DNA using a biolistic device is another route to prime specific CTL responses. Plasmid DNA is coated onto gold particles and delivered intradermally with a gene gun. Groups of mice (n=10) were immunised three times at two weeks intervals with the gene gun alone (4 µg/immunisation), immunised two times with the gene gun followed by an intravenous MVA.PbCSP boost or immunised intramuscularly with 50 µg of pTH.PbCSP and two weeks later boosted with MVA.PbCSP intravenously. Two weeks after the last immunisation the animals were challenged with 2000 sporozoites to assess protective efficacy of each immunisation regimen. In the group that received the intravenous MVA boost following two gene gun immunisations

one out of ten animals developed blood stage parasitemia (90% protection). Complete protection was observed with intramuscular DNA priming followed by MVA i.v boosting. Seven out of 10 animals that were immunised three times with the gene gun were infected. (30% protection)
 5 (Table 11).

Immunisation 1	Immunisation 2	Immunisation 3	No. inf./ No. chall.	% protection
DNA				
gene gun DNA	gene gun DNA	gene gun DNA	7/10	30
gene gun DNA	gene gun DNA	MVA.PbCSP	1/10	90
-	DNA i.m	MVA.PbCSP	0/10	100
Naïve			10/10	0

Table 11 Results of challenge experiments comparing different routes
 10 of DNA priming (intradermally by gene gun versus intramuscular needle injection). Groups of BALB/c mice (n=10) were immunised as indicated. Each gene gun immunisation delivered 4 µg of plasmid DNA intraepidermally. For i.m. immunisations 50 µg of plasmid DNA were injected. Twenty days after the last immunisation mice were challenged as
 15 described previously.

Highly susceptible C57BL/6 mice are protected

C57BL/6 mice are very susceptible to *P. berghei* sporozoite challenge. C57BL/6 mice were immunised using the DNA-MVA prime boost regime
 20 with both pre-erythrocytic antigens PbCSP and PbTRAP, and challenged with either 200 or 1000 infectious sporozoites per mouse. (Two hundred sporozoites corresponds to more than twice the dose required to induce infection in this strain). All ten mice challenged with 200 sporozoites showed sterile immunity. Even the group challenged with 1000

sporozoites, 60% of the mice were protected (Table 12). All the naïve C57BL/6 mice were infected after challenge.

Table 12 Protection of C57BL/6 mice from sporozoite challenge

	No. animals inf./ No. challenged	% protection
1000 sporozoites		
DNA followed by MVA	4/10	60
Naïve	5/5	0
200 sporozoites		
DNA followed by MVA	0/10	100
Naïve	5/5	0

Table 12 Results of a challenge experiment using C57BL/6 mice. Animals were immunised with PbCSP and PbTRAP using the DNA followed by MVA prime boost regime. Fourteen days later the mice were challenged with *P. berghei* sporozoites as indicated.

EXAMPLE 4

Protective efficacy of the DNA-priming/MVA-boosting regimen in two further disease models in mice

Following immunogenicity studies, the protective efficacy of the DNA-priming MVA-boosting regimen was tested in two additional murine challenge models. The two challenge models were the P815 tumour model and the influenza A virus challenge model. In both model systems CTL have been shown to mediate protection.

P815 tumour challenges:

Groups (n = 10) of DBA/2 mice were immunised with a combination of DNA followed by MVA expressing a tumour epitope string or the HM epitope

string. Two weeks after the last immunisation the mice were challenged intravenously with 10^5 P815 cells. Following this challenge the mice were monitored regularly for the development of tumour-related signs and survival.

5 Figure 13 shows the survival rate of the two groups of mice. Sixty days after challenge eight out of ten mice were alive in the group immunised with the tumour epitopes string. In the group immunised with the HM epitope string only 2 animals survived. This result is statistically significant: 2/10 vs 8/10 chi-squared = 7.2. $P = 0.007$. The onset of death
10 in the groups of animals immunised with the tumour epitope string is delayed compared to the groups immunised with the HM epitope string.

Influenza virus challenges:

Groups of BALB/c mice were immunised with three gene gun
15 immunisations with plasmid DNA, two intramuscular plasmid DNA injections, one i.m. DNA injection followed by one MVA.NP boost i.v. or two gene gun immunisations followed by one MVA.NP boost i.v. Plasmid DNA and recombinant MVA expressed the influenza virus nucleoprotein. Two weeks after the last immunisation the mice were challenged intranasally
20 with 100 HA of influenza A/PR/8/34 virus. The animals were monitored for survival daily after challenge.

Complete protection was observed in the following groups of animals

- two DNA gene gun immunisations followed by one MVA.NP boost i.v.,
- one i.m. DNA injection followed by one MVA.NP boost i.v.
- 25 • two i.m. DNA injections.

In the group of animals immunised three times with the gene gun 71% of the animals survived (5/7) and this difference from the control group was not significant statistically ($P > 0.05$). In the naive group 25% of
30 the animals survived (Figure 14) and this group differed significantly ($P < 0.05$) for the two completely protected groups.

Figure 14 shows results of an influenza virus challenge experiment. BALB/c mice were immunised as indicated. GG = gene gun immunisations, im = intramuscular injection, iv = intravenous injection. Survival of the animals was monitored daily after challenge.

5 In a second experiment groups of 10 BALB/c mice were immunised with MVA.NP i.v. alone, three times with the gene gun, two times with the gene gun followed by one MVA.NP boost i.v. and two i.m injections of V1J-NP followed by one MVA.NP boost. Two weeks after the last immunisation the mice were challenged with 100 HA units of influenza
10 A/PR/8/34 virus.

Complete and statistically significant protection was observed in the following groups of animals:

- two gene gun immunisations followed by one MVA.NP boost,
- two i.m injections of V1J-NP followed by one MVA.NP boost.

15 In the group receiving one MVA.NP i.v., 30% (3 out of 10) of animals survived. In the group immunised with a DNA vaccine delivered by the gene gun three times, 70% of the animals were protected but this protection was not significantly different from the naïve controls. In this challenge experiment 40% (4 out of 10) of the naive animals survived the
20 challenge.

EXAMPLE 5

Immunogenicity studies in non-human primates

25 **Immunogenicity and protective efficacy of the prime boost regimen in non-human primates.**

In order to show that the strong immunogenicity of the DNA priming/MVA boosting regime observed in mice translates into strong immunogenicity in primates, the regimen was tested in macaques. The
30 vaccine consisted of a string of CTL epitopes derived from HIV and SIV sequences (Figure 2), in plasmid DNA or MVA, denoted DNA.H and

MVA.H respectively. The use of defined CTL epitopes in a polypeptide string allows testing for SIV specific CTL in macaques. Due to the MHC class I restriction of the antigenic peptides, macaques were screened for their MHC class I haplotype and Mamu-A*01-positive animals were
5 selected for the experiments described.

Three animals (CYD, DI and DORIS) were immunised following this immunisation regimen:

	week 0	DNA (8µg, i.d., gene gun)
10	week 8	DNA (8µg, i.d., gene gun)
	week 17	MVA (5 x 10 ⁸ pfu, i.d.)
	week 22	MVA (5 x 10 ⁸ pfu, i.d.)

Blood from each animal was drawn at weeks 0, 2, 5, 8, 10,
15 11, 17, 18, 19, 21, 22, 23, 24 and 25 of the experiment. The animals were monitored for induction of CTL using two different methods. PBMC isolated from each bleed were re-stimulated *in vitro* with a peptide encoded in the epitope string and tested for their ability to recognise autologous peptide-loaded target cells in a chromium release cytotoxicity assay. Additionally,
20 freshly isolated PBMC were stained for antigen specific CD8+ T cells using tetramers.

Following two gene gun immunisations very low levels of CTL were detected using tetramer staining (Figure 15). Two weeks after the first MVA boosting, all three animals developed peptide specific CTL as
25 detected by tetramer staining (Figure 15). This was reflected by the detection of moderate CTL responses following *in vitro* restimulation (Figure 16, week 19). The second boost with MVA.H induced very high levels of CD8+, antigen specific T cells (Figure 15) and also very high levels of peptide specific cytotoxic T cells (Figure 16, week 23).

30 Figure 15 shows detection of SIV-specific MHC class I-restricted CD8+ T cells using tetramers. Three Mamu-A*A01-positive

macaques were immunised with plasmid DNA (gene gun) followed by MVA boosting as indicated. Frequencies of Mamu-A*01/CD8 double-positive T cells were identified following FACS analysis. Each bar represents the percentage of CD8+ T cells specific for the Mamu-A*01/gag epitope at the indicated time point. One percent of CD8 T cells corresponds to about 5000/10⁶ peripheral blood lymphocytes. Thus the levels of epitope-specific CD8 T cells in the peripheral blood of these macaques are at least as high as the levels observed in the spleens of immunised and protected mice in the malaria studies.

Figure 16 shows CTL induction in macaques following DNA/MVA immunisation. PBMC from three different macaques (CYD, DI and DORIS) were isolated at week 18, 19 and 23 and were restimulated with peptide CTPYDINQM [SEQ ID NO: 54] *in vitro*. After two restimulations with peptide CTPYDINQM [SEQ ID NO: 54] the cultures were tested for their lytic activity on peptide-pulsed autologous target cells. Strong CTL activity was observed.

EXAMPLE 6

Immunogenicity and Challenge Studies in Chimpanzees

To show that a similar regime of initial immunisation with plasmid DNA and subsequent immunisation with recombinant MVA can be effective against *Plasmodium falciparum* malaria in higher primates an immunisation and challenge study was performed with two chimpanzees. Chimp H1 received an initial immunisation with 500 µg of a plasmid expressing *Plasmodium falciparum* TRAP from the CMV promoter without intron A, CMV-TRAP. Chimp H2 received the same dose of CMV-LSA-1, which expresses the C-terminal portion of the LSA-1 gene of *P. falciparum*. Both chimps received three more immunisations over the next 2 months, but with three plasmids at each immunisation. H1 received CMV-TRAP as before, plus pTH-TRAP, which expresses TRAP using the CMV promoter

with intron A, leading to a higher expression level. H1 also received RSV-LSA-1, which expresses the C-terminal portion of LSA-1 from the RSV promoter. H2 received CMV-LSA-1, pTH-LSA-1 and RSV-TRAP at the second, third and fourth immunisations. The dose was always 500 µg of each plasmid.

It was subsequently discovered that the RSV plasmids did not express the antigens contained within them, so H1 was only immunised with plasmids expressing TRAP, and H2 with plasmids expressing LSA-1.

Between and following these DNA immunisations assays of cellular immune responses were performed at several time points, the last assay being performed at three months following the fourth DNA immunisation, but no malaria-specific T cells were detectable in either ELISPOT assays or CTL assays for CD8+ T cells.

Both animals were subsequently immunised with three doses of 10^8 ffu of a recombinant MVA virus encoding the *P. falciparum* TRAP antigen over a 6 week period. Just before and also following the third recombinant MVA immunisation T cell responses to the TRAP antigen were detectable in both chimpanzees using an ELISPOT assay to whole TRAP protein bound to latex beads. This assay detects both CD4+ and CD8+ T cell responses. Specific CD8+ T responses were searched for with a series of short 8-11 amino acid peptides in both immunised chimpanzees. Such analysis for CD8+ T cell responses indicated that CD8+ T cells were detectable only in the chimpanzee H1. The target epitope of these CD8+ T lymphocytes was an 11 amino acid peptide from TRAP, tr57, of sequence KTASCGVWDEW [SEQ ID NO: 78]. These CD8+ T cells from H1 had lytic activity against autologous target cells pulsed with the tr57 peptide and against autologous target cells infected with the recombinant PfTRAP-MVA virus. A high precursor frequency of these specific CD8+ T cells of about 1 per 500 lymphocytes was detected

in the peripheral blood of this chimpanzee H1 using an ELISPOT assay two months following the final MVA immunisation. No specific CD8+ T cell response was clearly detected in the chimpanzee H2, which was not primed with a plasmid DNA expressing TRAP.

5 Two months after the third PfTRAP-MVA immunisation challenge of H1 and H2 was performed with 20,000 sporozoites, a number that has previously been found to yield reliably detectable blood stage infection in chimpanzees 7 days after challenge (Thomas *et al.* 1994 and unpublished data). The challenge was performed with the NF54 strain of
10 *Plasmodium falciparum*. This is of importance because the TRAP sequence in the plasmid DNA and in the recombinant MVA is from the T9/96 strain of *P. falciparum* which has numerous amino acid differences to the NF54 TRAP allele (Robson *et al.* 1990). Thus, this sporozoite challenge was performed with a heterologous rather than homologous
15 strain of parasite. In the chimpanzee H2 parasites were detectable in peripheral blood as expected 7 days after sporozoite challenge using *in vitro* parasite culture detection. However, in H1 the appearance of blood stage parasites in culture from the day 7 blood samples was delayed by three days consistent with some immune protective effect against the liver-
20 stage infection. In studies of previous candidate malaria vaccines in humans a delay in the appearance of parasites in the peripheral blood has been estimated to correspond to a substantial reduction in parasite density in the liver (Davis *et al.* 1989). Thus the chimpanzee H1, immunised first with *P. falciparum* TRAP plasmid DNA and subsequently with the same
25 antigen expressed by a recombinant MVA virus showed a strong CD8+ T lymphocyte response and evidence of some protection from heterologous sporozoite challenge.

DISCUSSION

These examples demonstrate a novel regime for immunisation against malaria which induces high levels of protective CD8+ T cells in rodent models of human malaria infection. Also demonstrated is an unprecedented complete protection against sporozoite challenge using subunit vaccines (36 out of 36 mice protected in Table 6 using DNA priming and MVA boosting with the CS epitope containing vaccines). Induction of protective immune responses using the DNA priming/MVA boosting regimen was demonstrated in two additional mouse models of viral infection influenza A model and cancer (P815 tumour model). More importantly for vaccines for use in humans this immunisation regimen is also highly immunogenic for CD8+ T cells in primates. Strong SIV-gag-specific CTL were induced in 3 out of 3 macaques with plasmid DNA and MVA expressing epitope strings. The levels induced are comparable to those found in SIV-infected animals. The data from the chimpanzee studies indicate that the same immunisation regime can induce a strong CD8+ T lymphocyte response against *P. falciparum* in higher primates with some evidence of protection against *P. falciparum* sporozoite challenge.

Ty-VLPs have previously been reported to induce good levels of CD8+ T cell responses against the *P. berghei* rodent malaria (Allsopp *et al.* 1995) but alone this construct is not protective. It has now been found that subsequent immunisation with recombinant MVA boosts the CD8+ T cell response very substantially and generates a high level of protection (Table 7).

Recombinant MVA viruses have not been assessed for efficacy as malaria vaccines previously. Recombinant MVA alone was not significantly protective, nor was priming with recombinant MVA followed by a second immunisation with recombinant plasmid DNA. However, a second immunisation with the recombinant MVA following an initial immunisation with either Ty-VLPs or plasmid DNA yielded impressive

levels of protection. Non-recombinant MVA virus has been safely used to vaccinate thousands of human against smallpox and appears to have an excellent safety profile. The molecular basis of the increased safety and immunogenicity of this strain of vaccinia virus is being elucidated by
5 detailed molecular studies (Meyer *et al.* 1991; Sutter *et al.* 1994).

Plasmid DNA has previously been tested as a malaria vaccine for the *P. yoelii* rodent malaria. High levels of, but not complete, protection is seen in some strains but in other strains of mice little or no protection was observed even after multiple immunisations (Doolan *et al.*
10 1996). Although plasmid DNA has been proposed as a method of immunisation against *P. falciparum*, success has not previously been achieved. The evidence provided here is the first evidence to show that plasmid DNA may be used in an immunisation regime to induce protective immunity against the human malaria parasite *P. falciparum*.

15 A similar regime of immunisation to the regime demonstrated herein can be expected to induce useful protective immunity against *P. falciparum* in humans. It should be noted that five of the vaccine constructs employed in these studies to induce protective immunity in rodents or chimpanzees contain *P. falciparum* sequences and could
20 therefore be used for human immunisation against *P. falciparum*. These are: 1. The *P. falciparum* TRAP plasmid DNA vaccine. 2. The *P. falciparum* TRAP recombinant MVA virus. 3. The Ty-VLP encoding an epitope string of numerous *P. falciparum* epitopes, as well as the single *P. berghei* CTL epitope. 4. The plasmid DNA encoding the same epitope
25 string as 3. 5. The recombinant MVA encoding the longer HM epitope string including many of the malaria epitopes in 3 and 4. Similarly the plasmid DNAs and MVA encoding HIV epitopes for human class I molecules could be used in either prophylactic or therapeutic immunisation against HIV infection.

These studies have provided clear evidence that a novel sequential immunisation regime employing a non-replicating or replication-impaired pox virus as a boost is capable of inducing a strong protective CD8+ T cell response against the malaria parasite. The examples
5 demonstrate clearly a surprising and substantial enhancement of CD8+ T cell responses and protection compared to replicating strains of pox viruses. Because there is no reason to believe that the immunogenicity of CD8+ T cell epitopes from the malaria parasite should differ substantially from CD8+ T cell epitopes in other antigens it is expected that the
10 immunisation regime described herein will prove effective at generating CD8+ T cell responses of value against other diseases. The critical step in this immunisation regimen is the use of non-replicating or replication-impaired recombinant poxviruses to boost a pre-existing CTL response. We have shown that CTL responses can be primed using different antigen
15 delivery systems such as a DNA vaccine i.d. and i.m, a recombinant Ty-VLP, a recombinant adenovirus and irradiated sporozoites. This is supported by the data presented on the generation of a CD8+ T cell response against HIV, influenza virus and tumours. Amongst several known examples of other diseases against which a CD8+ T cell immune
20 response is important are the following: infection and disease caused by the viruses HIV, herpes simplex, herpes zoster, hepatitis C, hepatitis B, influenza, Epstein-Barr virus, measles, dengue and HTLV-1; by the bacteria *Mycobacterium tuberculosis* and *Listeria* sp.; and by the protozoan parasites *Toxoplasma* and *Trypanosoma*. Induction of protective CTL
25 responses against influenza A virus has been demonstrated in Figure 14. Furthermore, the immunisation regime described herein is expected to be of value in immunising against forms of cancer where CD8+ T cell responses plays a protective role. The induction of protective CTL responses using the DNA prime MVA boost regime against tumours is

shown in Figure 13. Specific examples in humans include melanoma, cancer of the breast and cancer of the colon.

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

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B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution <div style="margin-left: 20px;">European Collection of Animal Cell Cultures (CAMR)</div>	
Address of depositary institution (including postal code and country) <div style="margin-left: 20px;">Salisbury Wiltshire SP4 0JG United Kingdom</div>	
Date of deposit <div style="margin-left: 20px;">5 June 1997</div>	Accession Number <div style="margin-left: 20px;">V97060511</div>
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>	
In respect of all designated States to which such action is possible and to the extent that it is legally permissible under the law of the designated State, it is requested that a sample of the deposited microorganism be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), UK Patent Rules 1995, Schedule 2, Paragraph 3, Australian Regulation 3.25(3), Danish	
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

C. ADDITIONAL INDICATIONS (continued)

Patents Act Sections 22 and 33(3), Icelandic Patents Act Sections 22 and 33(3), Norwegian Patents Act Sections 22 and 33(3) and generally similar provisions *mutatis mutandis* for any other designated State.

CLAIMS

1. A kit for generating a protective CD8+ T cell immune
5 response against at least one target antigen, which kit comprises:
 - (i) a priming composition comprising a source of one or more CD8+ T cell epitopes of the target antigen, together with a pharmaceutically acceptable carrier; and
 - (ii) a boosting composition comprising a source of one or more CD8+ T
10 cell epitopes of the target antigen, including at least one CD8+ T cell epitope which is the same as a CD8+ T cell epitope of the priming composition, wherein the source of CD8+ T cell epitopes is a non-replicating or replication-impaired recombinant poxvirus vector, together with a pharmaceutically acceptable carrier;
- 15 with the proviso that if the source of epitopes in (i) is a viral vector, the viral vector in (ii) is derived from a different virus.
2. The kit according to claim 1, wherein the source of CD8+ T cell epitopes in (i) is a non-viral vector or a non-replicating or replication-impaired viral vector.
- 20 3. The kit according to claim 1 or claim 2, wherein the source of CD8+ T cell epitopes in (i) is not a poxvirus vector.
4. The kit according to claim 2 or claim 3, wherein the source of CD8+ T cell epitopes in (i) is DNA or RNA.
5. The kit according to claim 4, wherein the source of epitopes
25 in (i) is a recombinant DNA plasmid.
6. The kit according to claim 4 or claim 5, further comprising GM-CSF as an adjuvant for (i).
7. The kit according to any one of claims 1 to 6, wherein the
30 source of CD8+ T cell epitopes in (i) encodes or comprises the target antigen.

8. The kit according to any one of claims 4 to 6, wherein the source of epitopes in (i) encodes a single CD8+ T cell epitope or a recombinant string of two or more CD8+ T cell epitopes.
9. The kit according to any one of claims 1 to 3, wherein the
5 source of epitopes in (i) is a peptide, polypeptide, protein, polyprotein or particle comprising two or more CD8+ T cell epitopes, present in a recombinant string of CD8+ T cell epitopes or in a target antigen.
10. The kit according to claim 9, wherein the source of CD8+ T cell epitopes in (i) is a recombinant protein particle such as a Ty virus-like
10 particle (VLP).
11. The kit according to any one of claims 1 to 3, wherein the source of epitopes in (i) is a recombinant adenovirus vector.
12. The kit according to any one of claims 1 to 11, wherein the source of CD8+ T cell epitopes in (ii) is a recombinant vaccinia virus vector.
- 15 13. The kit according to claim 12, wherein the recombinant vaccinia virus vector is of the vaccinia virus strain Modified Virus Ankara (MVA), or a strain derived therefrom.
14. The kit according to claim 12, wherein the recombinant vaccinia virus vector is of the strain NYVAC or a strain derived therefrom.
- 20 15. The kit according to any one of claims 1 to 11, wherein the source of CD8+ T cell epitopes in (ii) is a recombinant avipox vector such as canary pox or fowl pox or strains derived therefrom such as ALVAC.
16. The kit according to any one of claims 1 to 15, for generating a protective immune response against a pathogen or tumour comprising
25 the target antigen.
17. The kit according to claim 16, for generating a protective immune response against a malaria pathogen such as *Plasmodium falciparum*.

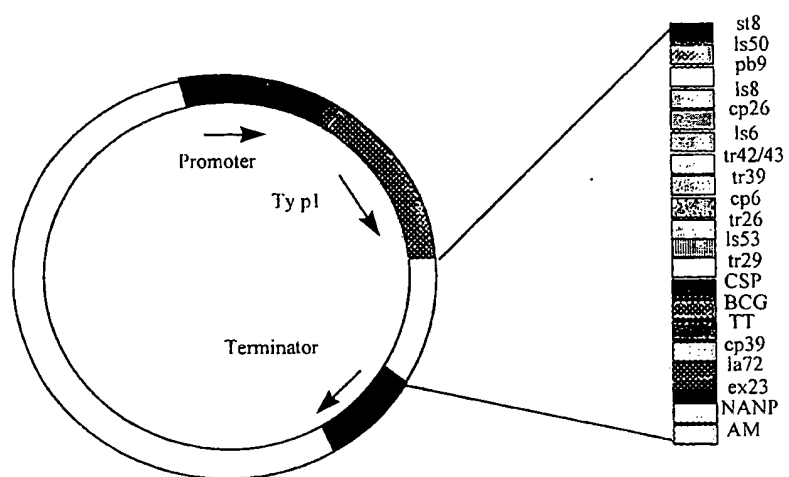
18. A kit according to claim 17, wherein the CD8+ T cell epitopes in or encoded by (i) include one or more malaria epitopes from the list given in table 1.
19. The kit according to claim 18, wherein the CD8+ T cell
5 epitopes in (i) include all of the epitopes given in table 1.
20. The kit according to claim 16, for generating an immune response against HIV.
21. The kit according to claim 20, wherein the CD8+ T cell epitopes in or encoded by (i) include one or more HIV epitopes from the list
10 given in table 2.
22. The kit according to claim 20, wherein the CD8+ T cell epitopes in or encoded by (i) include all of the epitopes given in table 2.
23. The kit according to any one of claims 1 to 22, wherein the priming and boosting compositions are identical in that both contain the
15 source of epitopes in (i) and the source of epitopes in (ii).
24. The kit according to any one of claims 1 to 23, wherein the priming composition and/or the boosting composition is in particulate form suitable for delivery by means of a gene gun.
25. A method for generating a protective CD8+ T cell immune
20 response against at least one target antigen, which method comprises administering at least one dose of component (i), followed by at least one dose of component (ii) of the kit according to any one of claims 1 to 24.
26. A method for generating a protective CD8+ T cell immune response against a pathogen or tumour, which method comprises
25 administering at least one dose of a recombinant DNA plasmid encoding at least one CD8+ T cell epitope or antigen of the pathogen or cancer, followed by at least one dose of a recombinant non-replicating or replication-impaired pox virus encoding the same epitope or antigen.
27. The method according to claim 25, wherein the recombinant
30 vaccinia virus is a recombinant MVA vector.

28. A method for generating a protective CD8+ T cell immune response against a pathogen or tumour, which method comprises administering at least one dose of a recombinant protein or particle comprising at least one epitope or antigen of the pathogen or cancer,
5 followed by at least one dose of a recombinant MVA vector encoding the same epitope or antigen.
29. The method according to any one of claims 26 to 28, for generating a protective CD8+ T cell immune response against malaria such as *P. falciparum* malaria.
- 10 30. The method according to any one of claims 26 to 28, for generating a protective CD8+ T cell immune response against HIV.
31. The method according to any one of claims 25 to 30, wherein (ii) is delivered intravenously, intraepidermally or intradermally.
32. A medicament for boosting a primed CD8+ T cell response
15 against at least one target antigen, comprising a source of one or more CD8+ T cell epitopes of the target antigen, wherein the source of CD8+ T cell epitopes is a non-replicating or a replication-impaired recombinant poxvirus vector, and a pharmaceutically acceptable carrier.
33. The medicament according to claim 32, wherein the vector is
20 a vaccinia virus vector such as MVA.
34. The medicament according to claim 32 or 33, for boosting a naturally primed CD8+ T cell response against malaria.
35. A method of boosting a primed CD8+ T cell immune response, which method comprises administering a medicament according
25 to any one of claims 32 to 34.
36. The use of a recombinant non-replicating or replication-impaired pox virus vector in the manufacture of a medicament for boosting a CD8+ T cell immune response.
37. The use of an MVA vector in the manufacture of a
30 medicament for boosting a CD8+ T cell immune response.

38. An epitope string comprising the amino acid sequences listed in table 1.
39. A recombinant Ty-VLP comprising the epitope string according to claim 38, for immunising against malaria.
- 5 40. A recombinant DNA plasmid or recombinant non-replicating or replication-impaired pox virus encoding the epitope string according to claim 38, for immunising against malaria.
41. A recombinant DNA plasmid or recombinant non-replicating or replication-impaired pox virus encoding the *P. falciparum* antigen TRAP, for immunising against malaria.
- 10 42. A recombinant vaccinia virus according to claim 40 or claim 41, of the MVA strain.
43. An epitope string comprising the amino acid sequences listed in table 2.
- 15 44. A recombinant polypeptide comprising a whole or substantially whole protein antigen such as TRAP and a string of two or more epitopes such as CTL epitopes from malaria.

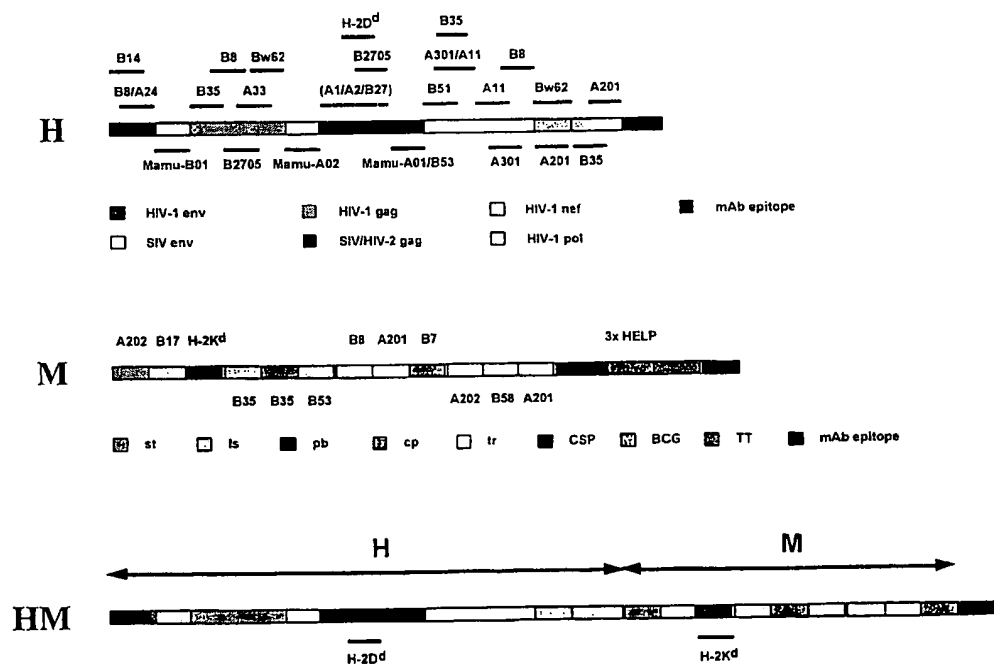
1/13

Figure 1



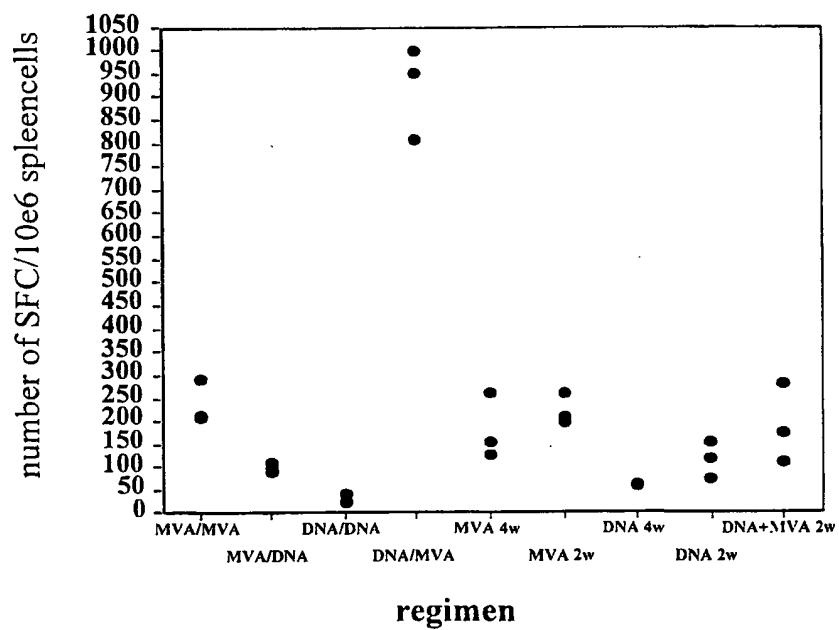
2/13

Figure 2



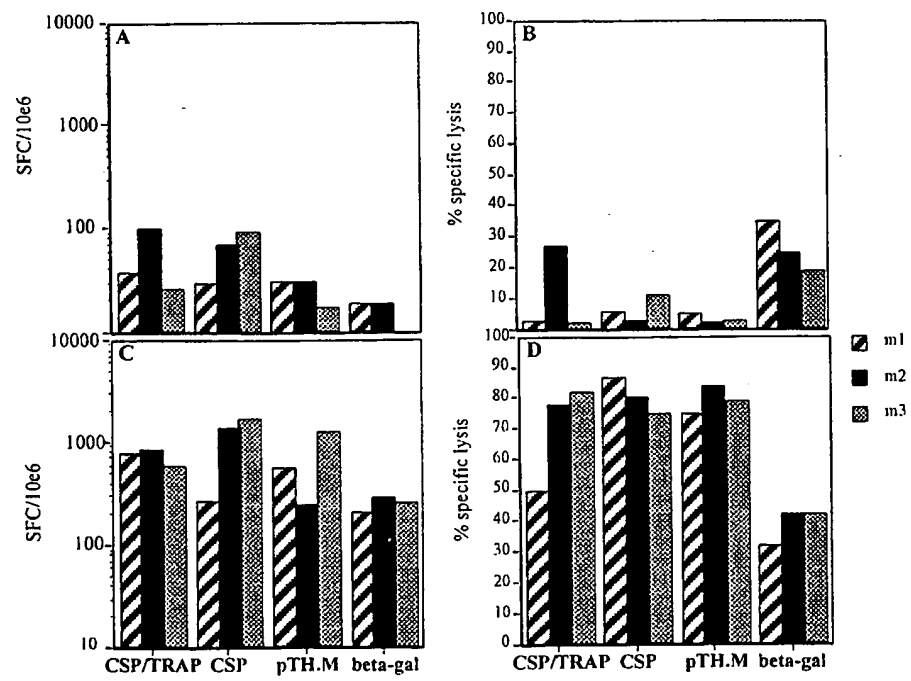
3/13

Figure 3



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Figure 4



5/13

Figure 5

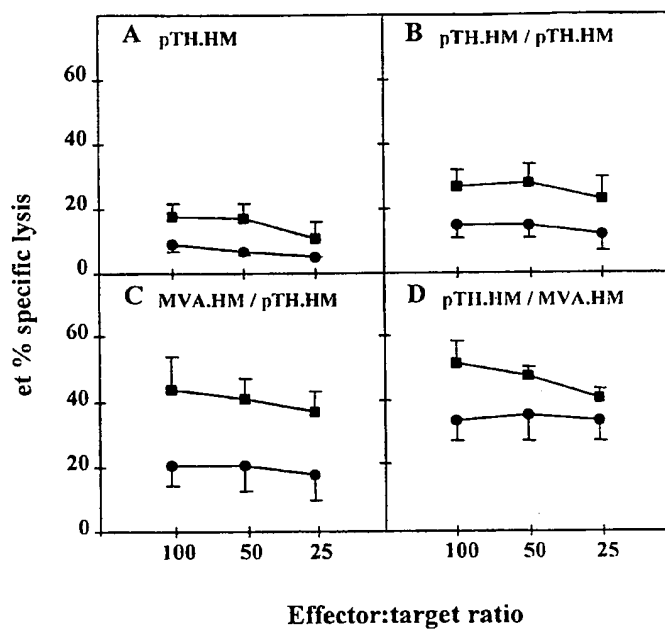
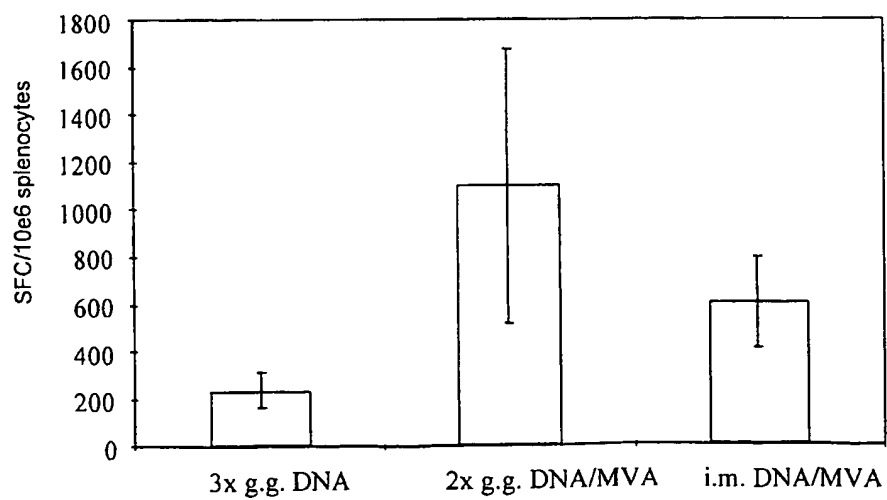
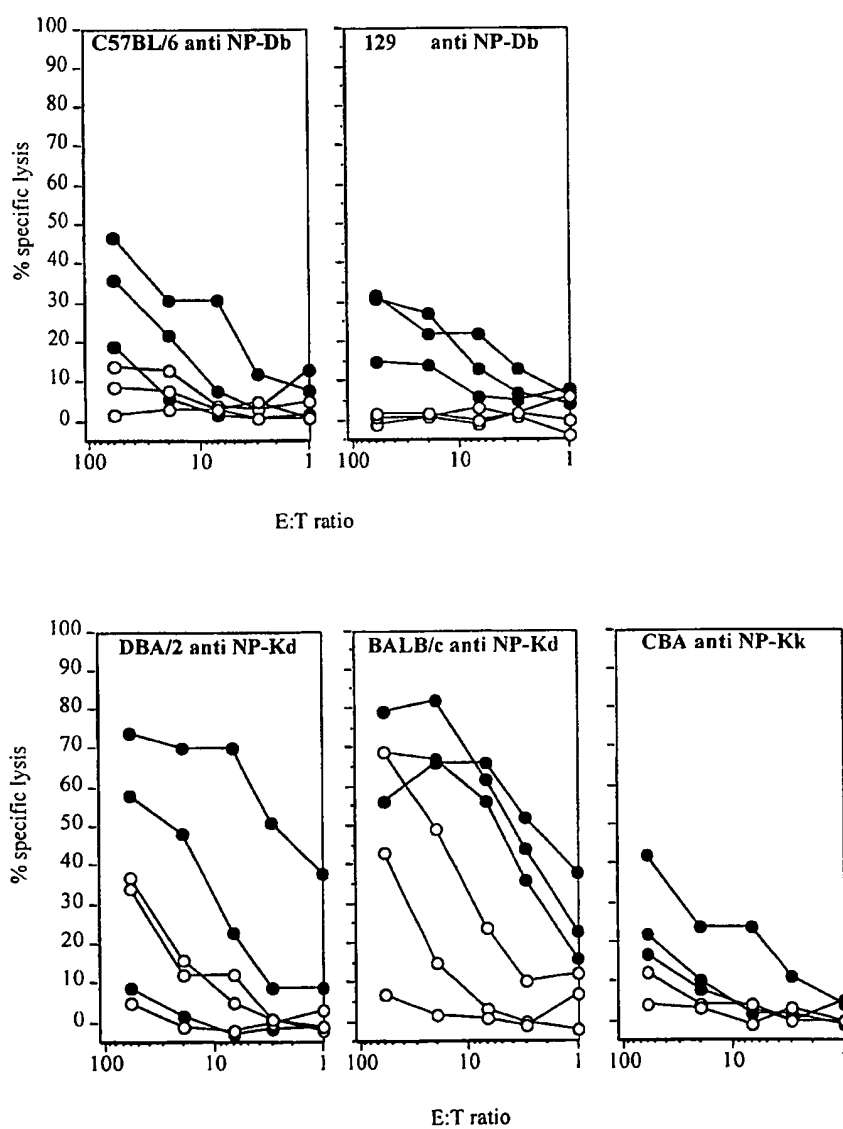


Figure 6



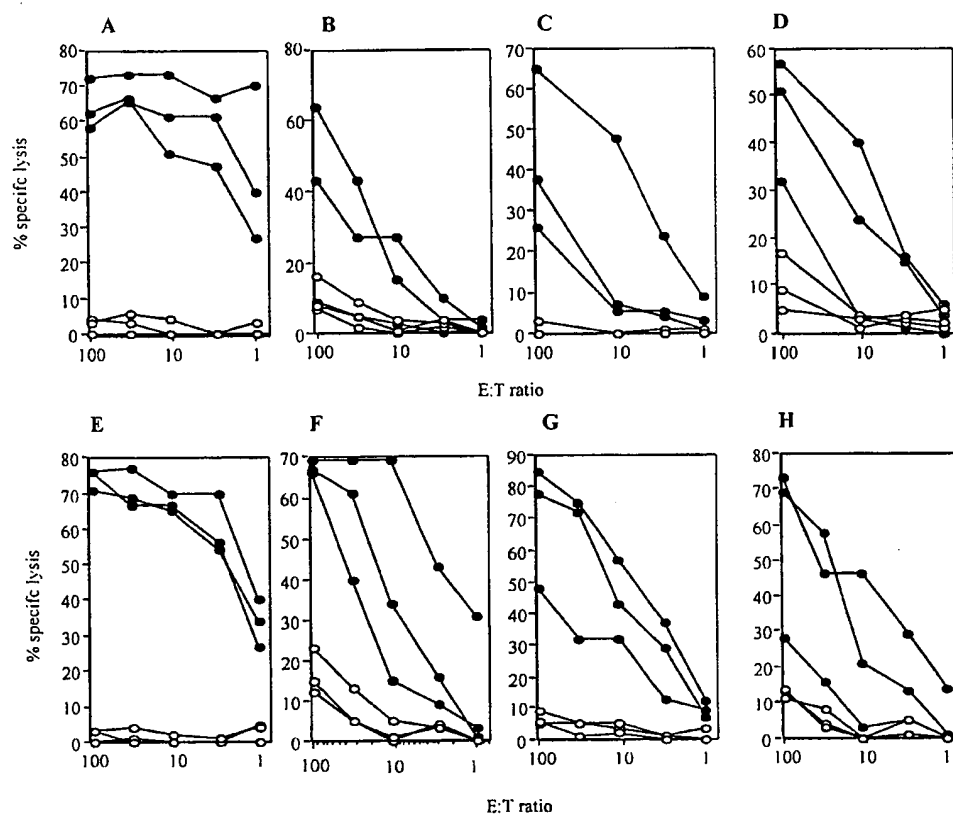
6/13

Figure 7



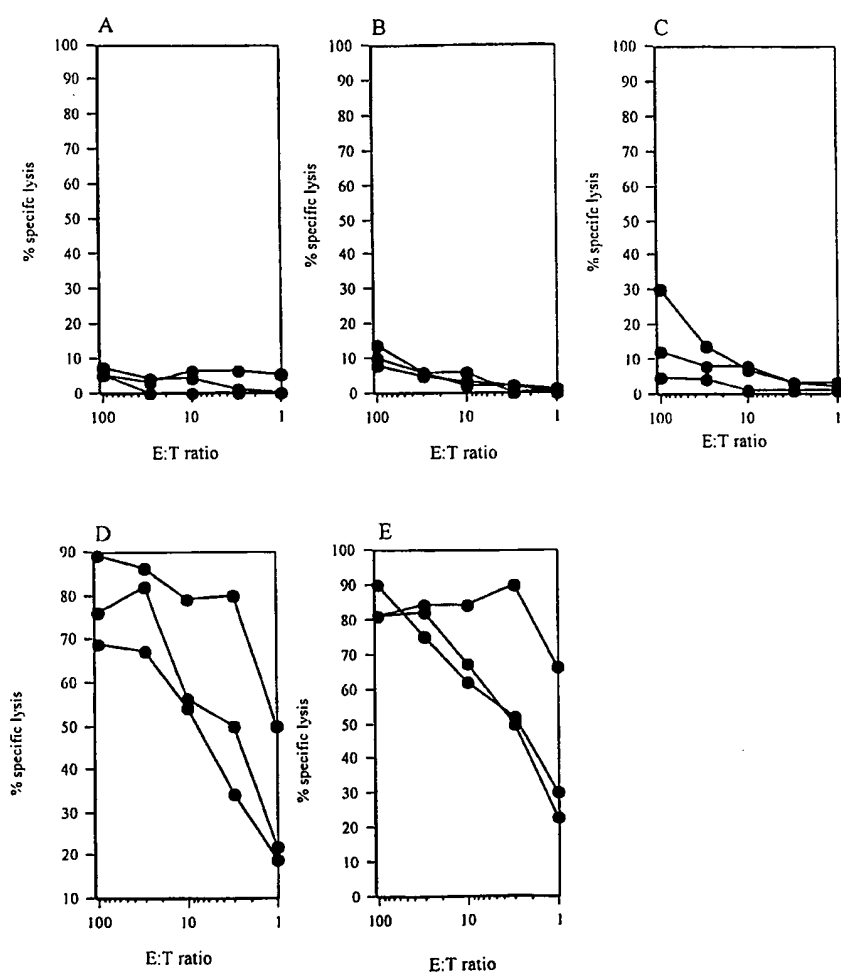
7/13

Figure 8



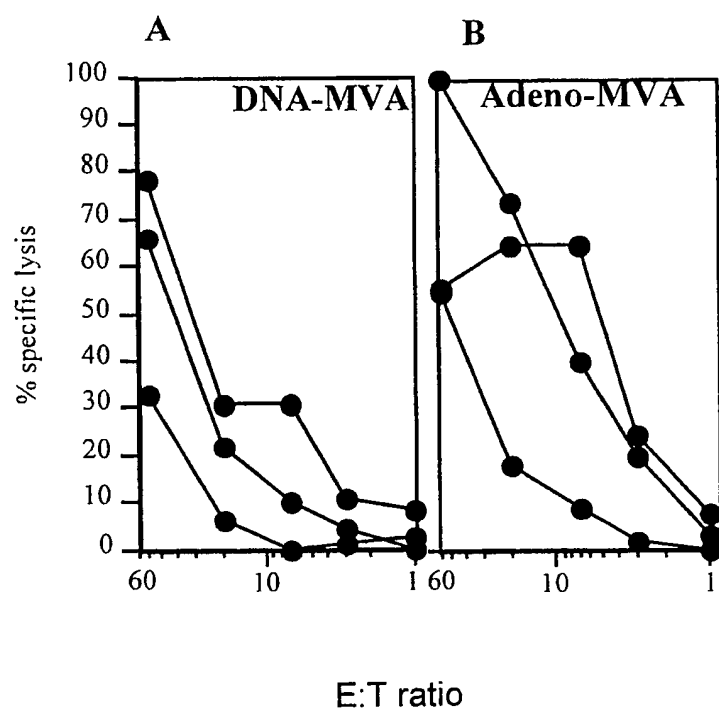
8/13

Figure 9



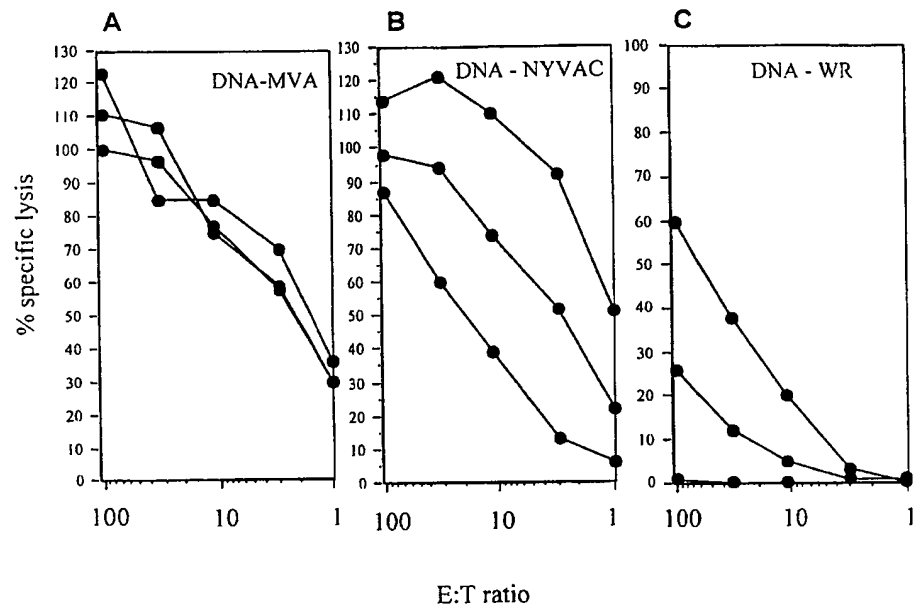
9/13

Figure 10



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Figure 11



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Figure 12

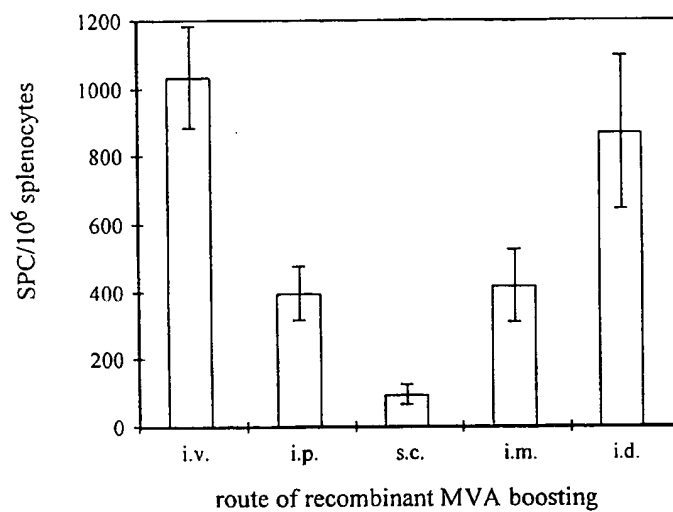
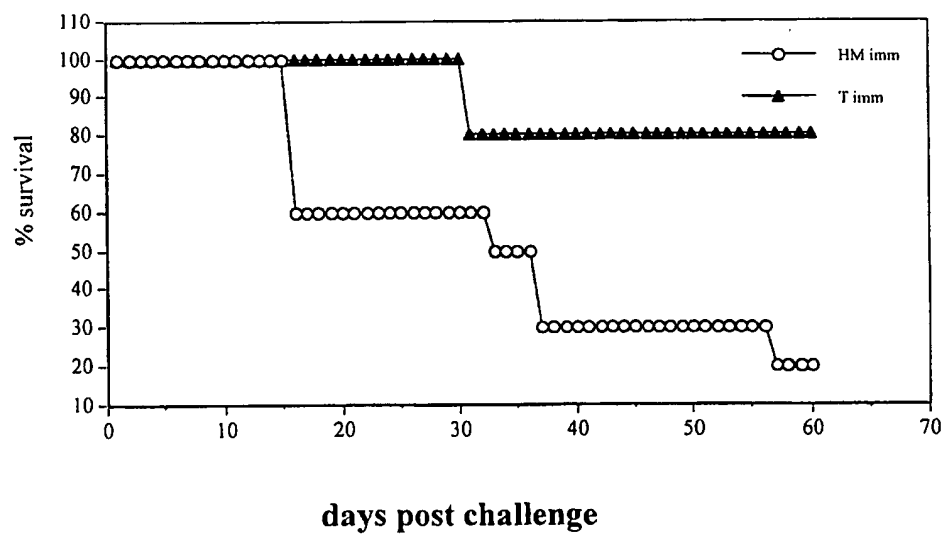
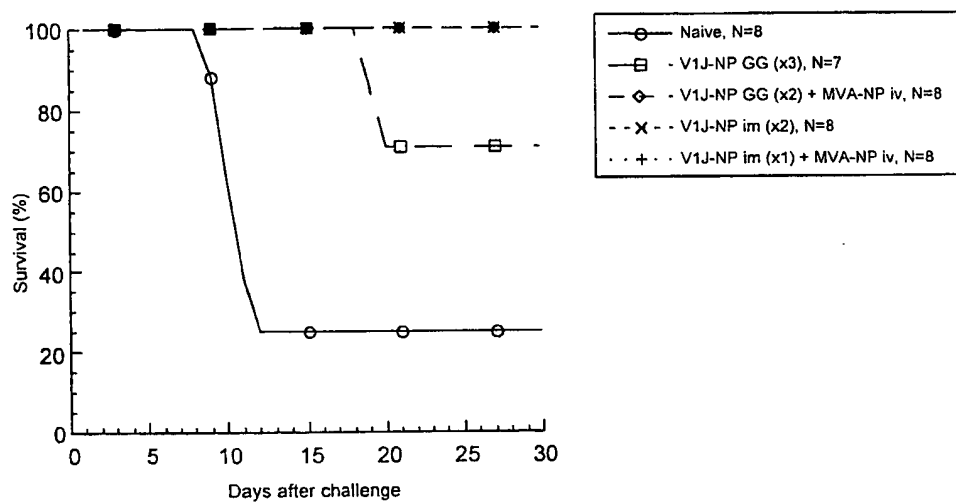


Figure 13



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Figure 14



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Figure 15

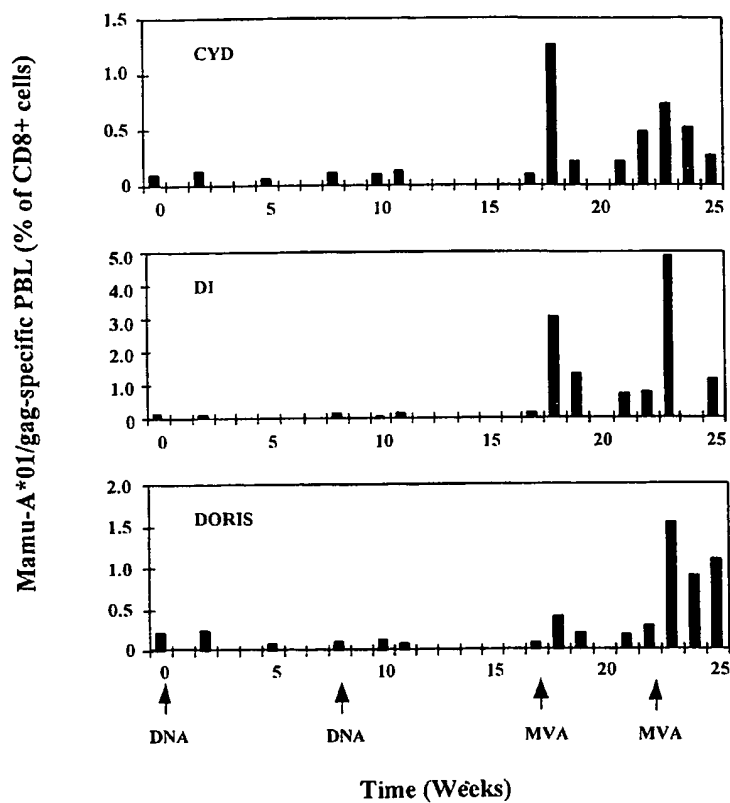
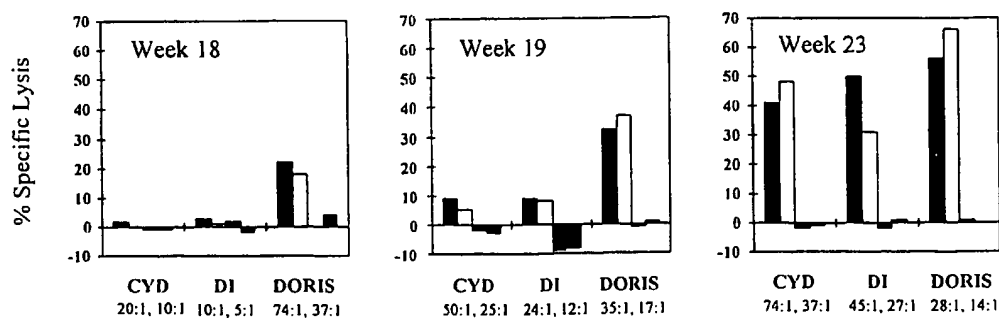


Figure 16



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/30, 15/48, 15/86, A61K 45/00, 39/015, 39/21		A3	(11) International Publication Number: WO 98/56919 (43) International Publication Date: 17 December 1998 (17.12.98)
(21) International Application Number: PCT/GB98/01681 (22) International Filing Date: 9 June 1998 (09.06.98) (30) Priority Data: 9711957.2 9 June 1997 (09.06.97) GB (71) Applicant (for all designated States except US): ISIS INNOVATION LIMITED [GB/GB]; 2 South Parks Road, Oxford OX1 3UB (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): McMICHAEL, Andrew, James [GB/GB]; Midsummer Cottage, Church Street, Beckley, Oxford OX3 9UT (GB). HILL, Adrian, Vivian, Sinton [IE/GB]; 2 St. Andrew's Road, Oxford OX3 9DL (GB). GILBERT, Sarah, Catherine [GB/GB]; 65 Dene Road, Headington, Oxford OX3 7EQ (GB). SCHNEIDER, Joerg [DE/GB]; 11 Malford Road, Barton, Oxford OX3 8BT (GB). PLEBANSKI, Magdalena [MX/GB]; Nuffield Dept. of Medicine, John Radcliffe Hospital, Oxford OX3 9DU (GB). HANKE, Tomas [CA/GB]; Molecular Immunology Group, Institute of Molecular Medicine, Headington, Oxford OX3 9DS (GB). SMITH, Geoffrey, Lilley [GB/GB]; University of Oxford, Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE (GB). BLANCHARD,		Tom [GB/GM]; MRC Laboratories Fajara, P.O. Box 273, Banjul (GM). (74) Agent: PRIVETT, Kathryn, L.; Stevens, Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 17 June 1999 (17.06.99)	
(54) Title: METHODS AND REAGENTS FOR VACCINATION WHICH GENERATE A CD8 T CELL IMMUNE RESPONSE			
(57) Abstract New methods and reagents for vaccination are described which generate a CD8 T cell immune response against malarial and other antigens such as viral and tumour antigens. Novel vaccination regimes are described which employ a priming composition and a boosting composition, the boosting composition comprising a non-replicating or replication-impaired pox virus vector carrying at least one CD8 T cell epitope which is also present in the priming composition.			

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/01681

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/30 C12N15/48 C12N15/86 A61K45/00 A61K39/015
A61K39/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>HODGE, JAMES W. ET AL: "Diversified prime and boost protocols using recombinant vaccini virus and recombinant non-replicating avian pox virus to enhance T-cell immunity and antitumor responses" VACCINE (APRIL-MAY 1997), 15(6/7), 759-768 CODEN: VACCDE;ISSN: 0264-410X, XP004064540 see page 759 see page 765, right-hand column - page 766, left-hand column see page 767, right-hand column --- -/--</p>	<p>1,4,7,8, 15,16, 25,26, 31-33, 35,36</p>

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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Date of the actual completion of the international search

16 March 1999

Date of mailing of the international search report

12. 04. 99

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/01681

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>AIDOO, MICHAEL ET AL: "Recombinant vaccinia viruses for the characterization of Plasmodium falciparum-specific cytotoxic T lymphocytes: recognition of processed antigen despite limited re-stimulation efficacy" INT. IMMUNOL. (MAY 1997), 9(5), 731-737 CODEN: INIMEN;ISSN: 0953-8178, XP002096556 see page 731</p> <p style="text-align: center;">---</p>	<p>1-19, 22-29, 31-42,44</p>
A	<p>CARROLL, MILES W. ET AL: "Highly attenuated modified vaccinia virus Ankara (MVA) as an effective recombinant vector: A murine tumor model." VACCINE, (MARCH 1997) VOL. 15, NO. 4, PP. 387-394. ISSN: 0264-410X., XP004094431 see page 387</p> <p style="text-align: center;">---</p>	<p>13,27, 28,33, 37,42</p>
A	<p>LIMBACH, K. J. ET AL: "Non - replicating expression vectors: Applications in vaccine development and gene therapy." EPIDEMIOLOGY AND INFECTION, (1996) VOL. 116, NO. 3, PP. 241-256. ISSN: 0950-2688., XP002096557 see page 241 - page 246, left-hand column</p> <p style="text-align: center;">---</p>	<p>1-37, 40-43</p>
A	<p>LAYTON, G. T. ET AL: "Induction of single and dual cytotoxic T-lymphocyte responses to viral proteins in mice using recombinant hybrid Ty - virus - like particles" IMMUNOLOGY (1996), 87(2), 171-8 CODEN: IMMUM;ISSN: 0019-2805, XP002096558 see page 171 - page 172, left-hand column</p> <p style="text-align: center;">---</p>	<p>10,39</p>
T	<p>TSANG K Y ET AL: "Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine 'see comments!'" JOURNAL OF THE NATIONAL CANCER INSTITUTE, (1995 JUL 5) 87 (13) 982-90. JOURNAL CODE: J9J. ISSN: 0027-8874., XP000578019 United States see page 982 see page 985, right-hand column - page 987; table 3</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	<p>1,4,7,8, 15,16, 25,26, 31-33, 35,36</p>

INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	BLANCHARD, TOM J. ET AL: "Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine" J. GEN. VIROL. (MAY 1998), 79(5), 1159-1167 CODEN: JGVIAY;ISSN: 0022-1317, XP002096559 see page 1159 see page 1167, left-hand column ---	13,27, 28,33, 37,42
P,X	HANKE T ET AL: "Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime." VACCINE, (1998 MAR) 16 (5) 439-45. JOURNAL CODE: X60. ISSN: 0264-410X., XP004106957 ENGLAND: United Kingdom see the whole document ---	1-44
P,X	SCHNEIDER, JORG ET AL: "Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara" NAT. MED. (N. Y.) (APRIL 1998), 4(4), 397-402 CODEN: NAMEFI;ISSN: 1078-8956, XP000739989 see page 397 see page 401 - page 402 ---	1-44
P,X	HANKE, T. ET AL: "Immunogenicities of intravenous and intramuscular administrations of modified vaccinia virus Ankara -base multi-CTL epitope vaccine for human immunodeficiency virus type 1 in mice" J. GEN. VIROL. (JANUARY 1998), 79(1), 83-90 CODEN: JGVIAY;ISSN: 0022-1317, XP002096554 see page 83 see page 87, right-hand column - page 88 ---	1-44
P,X	HANKE T ET AL: "DNA multi - CTL epitope vaccines for HIV and Plasmodium falciparum: immunogenicity in mice." VACCINE, (1998 FEB) 16 (4) 426-35. JOURNAL CODE: X60. ISSN: 0264-410X., XP004099305 ENGLAND: United Kingdom see the whole document ---	1-44

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INTERNATIONAL SEARCH REPORT

Internat'l Application No

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>GILBERT, SARAH C. ET AL: "A protein particle vaccine containing multiple malaria epitopes"</p> <p>NAT. BIOTECHNOL. (NOVEMBER 1997), 15(12), 1280-1284 CODEN: NABIF9; ISSN: 1087-0156, XP002096555</p> <p>see page 1280</p> <p>see page 1281; table 1</p> <p>-----</p>	1-44

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/01681

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 25-31 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the priming and boosting compositions.
2. ☒ Claims Nos.: 1-43, 44 (partly)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 98 01681

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 1-43, 44 (partly)

Due to the use of the term "such" which has no limiting effect on the scope of the claim, the subject-matter of claim 44 encompasses any recombinant peptide comprising any whole or substantially whole protein antigen and a string of two or more epitopes of any antigen. Therefore, said subject-matter is so vague and so indefinite that no complete meaningful search can be carried out. Only those embodiments of said subject-matter which comprise TRAP and a string of two or more CTL epitopes of a malaria antigen have been searched.



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification⁶ : C12N 15/30, A61K 45/00, 39/015</p>	<p>A2</p>	<p>(11) International Publication Number: WO 98/56919 (43) International Publication Date: 17 December 1998 (17.12.98)</p>
<p>(21) International Application Number: PCT/GB98/01681 (22) International Filing Date: 9 June 1998 (09.06.98) (30) Priority Data: 9711957.2 9 June 1997 (09.06.97) GB (71) Applicant (for all designated States except US): ISIS INNOVATION LIMITED [GB/GB]; 2 South Parks Road, Oxford OX1 3UB (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): McMICHAEL, Andrew, James [GB/GB]; Midsummer Cottage, Church Street, Beckley, Oxford OX3 9UT (GB). HILL, Adrian, Vivian, Sinton [IE/GB]; 2 St. Andrew's Road, Oxford OX3 9DL (GB). GILBERT, Sarah, Catherine [GB/GB]; 65 Dene Road, Headington, Oxford OX3 7EQ (GB). SCHNEIDER, Joerg [DE/GB]; 11 Malford Road, Barton, Oxford OX3 8BT (GB). PLEBANSKI, Magdalena [MX/GB]; Nuffield Dept. of Medicine, John Radcliffe Hospital, Oxford OX3 9DU (GB). HANKE, Tomas [CA/GB]; Molecular Immunology Group, Institute of Molecular Medicine, Headington, Oxford OX3 9DS (GB). SMITH, Geoffrey, Lilley [GB/GB]; University of Oxford, Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE (GB). BLANCHARD,</p>		<p>Tom [GB/GM]; MRC Laboratories Fajara, P.O. Box 273, Banjul (GM). (74) Agent: PRIVETT, Kathryn, L.; Stevens, Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i></p>
<p>(54) Title: METHODS AND REAGENTS FOR VACCINATION WHICH GENERATE A CD8 T CELL IMMUNE RESPONSE (57) Abstract New methods and reagents for vaccination are described which generate a CD8 T cell immune response against malarial and other antigens such as viral and tumour antigens. Novel vaccination regimes are described which employ a priming composition and a boosting composition, the boosting composition comprising a non-replicating or replication-impaired pox virus vector carrying at least one CD8 T cell epitope which is also present in the priming composition.</p>		

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METHODS AND REAGENTS FOR VACCINATION WHICH GENERATE A CD8 T CELL IMMUNE RESPONSE

This invention relates to generation of a protective CD8+ T cell immune response against target antigens using different primer and
5 booster compositions as sources of CD8+ T cell epitopes.

Introduction

A general problem in vaccinology has been an inability to generate high levels of CD8 T cells by immunisation. This has impeded
10 the development of vaccines against several diseases including malaria.

Plasmodium falciparum malaria causes hundreds of millions of malaria infections each year and is responsible for 1-2 million deaths annually. The development of an effective vaccine against malaria is thus a major priority for global public health. A considerable body of
15 immunological research over the last twenty years had led to the identification both of candidate vaccine antigens from the parasite and immunological mechanisms on the host that are likely to protect against infection and disease. However, despite this progress there is still no means of vaccinating against malaria infection which has been shown to
20 be effective in field trials.

A major problem has been the identification of a means of inducing a sufficiently strong immune response in vaccinated individuals to protect against infection and disease. So, although many malaria antigens are known that might be useful in vaccinating against malaria the problem
25 has been how to deliver such antigens or fragments of them known as epitopes, which are recognised by cells of the immune system, in a way that induces a sufficiently strong immune response of a particular type.

It has been known for many years that it is possible to protect individuals by immunising them with very large doses of irradiated malaria
30 sporozoites given by bites from infected mosquitoes. Although this is a

wholly impractical method of mass vaccination it has provided a model for analysing the immune responses that might be mediating protective immunity against sporozoite infection (Nardin and Nussenzweig 1993).

A considerable amount of research over the last decade or
5 more has indicated that a major protective immune response against the early pre-erythrocytic stage of *P. falciparum* malaria is mediated by T lymphocytes of the CD8+ ve type (CD8+ T cells). Such cells have been shown to mediate protection directly in mouse models of malaria infection (Nardin and Nussenzweig 1993). Such T cells have also been identified in
10 individuals naturally exposed to malaria and in volunteers immunised with irradiated sporozoites (Hill *et al.* 1991; Aidoo *et al.* 1995; Wizen *et al.* 1995). There is much indirect evidence that such CD8+ T cells are protective against malaria infection and disease in humans (Lalvani *et al.* 1994).

CD8+ T cells may function in more than one way. The best
15 known function is the killing or lysis of target cells bearing peptide antigen in the context of an MHC class I molecule. Hence these cells are often termed cytotoxic T lymphocytes (CTL). However, another function, perhaps of greater protective relevance in malaria infections is the ability of CD8+ T cells to secrete interferon gamma (IFN- γ). Thus assays of lytic activity and
20 of IFN- γ release are both of value in measuring a CD8+ T cell immune response. In malaria these CD8+ve cells can protect by killing the parasite at the early intrahepatic stage of malaria infection before any symptoms of disease are produced (Seguin *et al.* 1994) .

The agent of fatal human malaria, *P. falciparum* infects a
25 restricted number of host species: humans, chimpanzees and some species of New World monkey. The best non-human model of malaria is the chimpanzee because this species is closely related to humans and liver-stage infection is observed consistently unlike in the monkey hosts (Thomas *et al.* 1994). Because of the expense and limited availability of
30 chimpanzees most laboratory studies of malaria are performed in mice,

using the rodent malaria species *P. berghei* or *P. yoelii*. These latter two models are well studied and it has been shown in both that CD8+ve lymphocytes play a key role in protective immunity against sporozoite challenge.

5 Previous studies have assessed a large variety of means of inducing CD8+ T cell responses against malaria. Several of these have shown some level of CD8+ T cell response and partial protection against malaria infection in the rodent models (e.g. Li *et al.* 1993; Sedegah *et al.* 1994; Lanar *et al.* 1996). However, an effective means of immunising with
10 subunit vaccines by the induction of sufficiently high levels of CD8+ T lymphocytes to protect effectively against malaria sporozoite infection has not previously been demonstrated.

 In recent years improved immune responses generated to potential vaccines have been sought by varying the vectors used to deliver
15 the antigen. There is evidence that in some instances antibody responses are improved by using two different vectors administered sequentially as prime and boost. A variety of combinations of prime and boost have been tested in different potential vaccine regimes.

 Leong *et al.* (Vaccines 1995, 327-331) describe immunising
20 mice firstly to DNA expressing the influenza haemagglutinin (HA) antigen and then with a recombinant fowlpox vector expressing HA. An enhanced antibody response was obtained following boosting.

 Richmond *et al.* (Virology 1997, 230: 265-274) describe attempts to raise neutralising antibodies against HIV-1 env using DNA
25 priming and recombinant vaccinia virus boosting. Only low levels of antibody responses were observed with this prime boost regime and the results were considered disappointing.

 Fuller *et al.* (Vaccine 1997, 15:924-926 and Immunol Cell Biol 1997, 75:389-396) describe an enhancement of antibody responses to
30 DNA immunisation of macaques by using a booster immunisation with

replicating recombinant vaccinia viruses. However, this did not translate into enhanced protective efficacy as a greater reduction in viral burden and attenuation of CD4 T cell loss was seen in the DNA primed and boosted animals.

5 Hodge *et al* (Vaccine 1997, 15: 759-768) describe the induction of lymphoproliferative T cell responses in a mouse model for cancer using human carcinoembryonic antigen (CEA) expressed in a recombinant fowl pox virus (ALVAC). The authors primed an immune response with CEA-recombinant replication competent vaccinia viruses of
10 the Wyeth or WR strain and boosted the response with CEA-recombinant ALVAC. This led to an increase in T cell proliferation but did not result in enhanced protective efficacy if compared to three wild type recombinant immunisations (100% protection), three recombinant ALVAC-CEA immunisations (70% protection) or WR prime followed by two ALVAC-CEA
15 immunisations (63% protection).

 Thus some studies of heterologous prime-boost combination have found some enhancement of antibody and lymphoproliferative responses but no significant effect on protective efficacy in an animal model. CD8 T cells were not measured in these studies. The limited
20 enhancement of antibody response probably simply reflects the fact that antibodies to the priming immunogen will often reduce the immunogenicity of a second immunisation with the same immunogen, while boosting with a different carrier will in part overcome this problem. This mechanism would not be expected to be significantly affected by the order of immunisation.

25 Evidence that a heterologous prime boost immunisation regime might affect CD8 T cell responses was provided by Li *et al.* (1993). They described partial protective efficacy induced in mice against malaria sporozoite challenge by administering two live viral vectors, a recombinant replicating influenza virus followed by a recombinant replicating vaccinia
30 virus encoding a malaria epitope. Reversing the order of immunisation led

to loss of all protective efficacy and the authors suggested that this might be related to infection of liver cells by vaccinia, resulting in localisation of CTLs in the liver to protect against the hepatocytic stages of malaria parasites.

5 Rodrigues *et al.* (J. Immunol. 1994, 4636-4648) describe immunising mice with repeated doses of a recombinant influenza virus expressing an immunodominant B cell epitope of the malarial circumsporozoite (CS) protein followed by a recombinant vaccinia virus booster. The use of a wild type vaccinia strain and an attenuated but
10 replication-competent vaccinia strain in the booster yielded very similar levels of partial protection. However the attenuated but replication competent strain was slightly less immunogenic for priming CD8 T cells than the wild type vaccinia strain.

 Murata *et al.* (Cell. Immunol. 1996, 173: 96-107) reported
15 enhanced CD8 T cell responses after priming with replicating recombinant influenza viruses and boosting with a replicating strain of vaccinia virus and suggested that the partial protection observed in the two earlier studies was attributable to this enhanced CD8 T cell induction.

 Thus these three studies together provide evidence that a
20 booster immunisation with a replicating recombinant vaccinia virus may enhance to some degree CD8 T cell induction following priming with a replicating recombinant influenza virus. However, there are two limitations to these findings in terms of their potential usefulness. Firstly, the immunogenicity induced was only sufficient to achieve partial protection
25 against malaria and even this was dependent on a highly immunogenic priming immunisation with an unusual replicating recombinant influenza virus. Secondly, because of the potential and documented side-effects of using these replicating viruses as immunogens these recombinant vectors are not suitable for general human use as vaccines.

Modified vaccinia virus Ankara (MVA) is a strain of vaccinia virus which does not replicate in most cell types, including normal human tissues. MVA was derived by serial passage >500 times in chick embryo fibroblasts (CEF) of material derived from a pox lesion on a horse in
5 Ankara, Turkey (Mayr *et al.* 1975). It was shown to be replication-impaired yet able to induce protective immunity against veterinary poxvirus infections (Mayr 1976). MVA was used as a human vaccine in the final stages of the smallpox eradication campaign, being administered by intracutaneous, subcutaneous and intramuscular routes to >120,000
10 subjects in southern Germany. No significant side effects were recorded, despite the deliberate targeting of vaccination to high risk groups such as those with eczema (Mayr *et al.* 1978; Stickl *et al.* 1974; Mahnel *et al.* 1994;). The safety of MVA reflects the avirulence of the virus in animal models, including irradiated mice and following intracranial administration
15 to neonatal mice. The non-replication of MVA has been correlated with the production of proliferative white plaques on chick chorioallantoic membrane, abortive infection of non-avian cells, and the presence of six genomic deletions totalling approximately 30 kb (Meyer *et al.* 1991). The avirulence of MVA has been ascribed partially to deletions affecting host
20 range genes K1L and C7L, although limited viral replication still occurs on human TK-143 cells and African Green Monkey CV-1 cells (Altenburger *et al.* 1989). Restoration of the K1L gene only partially restores MVA host range (Sutter *et al.* 1994). The host range restriction appears to occur during viral particle maturation, with only immature virions being observed
25 in human HeLa cells on electron microscopy (Sutter *et al.* 1992). The late block in viral replication does not prevent efficient expression of recombinant genes in MVA. Recombinant MVA expressing influenza nucleoprotein, influenza haemagglutinin, and SIV proteins have proved to be immunogenic and provide varying degrees of protection in animal
30 models, although this has never been ascribed to CD8+ T lymphocytes

alone (Sutter *et al.* 1994, Hirsch *et al.* 1995; Hirsch *et al.* 1996).

Recombinant MVA is considered a promising human vaccine candidate because of these properties of safety and immunogenicity (Moss *et al.* 1995). Recombinant MVA containing DNA which codes for foreign
5 antigens is described in US 5,185,146 (Altenburger).

Poxviruses have evolved strategies for evasion of the host immune response that include the production of secreted proteins that function as soluble receptors for tumour necrosis factor, IL-1 β , interferon (IFN)- α/β and IFN- γ , which normally have sequence similarity to the
10 extracellular domain of cellular cytokine receptors (Symons *et al.* 1995; Alcamì *et al.* 1995; Alcamì *et al.* 1992). The most recently described receptor of this nature is a chemokine receptor (Graham *et al.* 1997). These viral receptors generally inhibit or subvert an appropriate host immune response, and their presence is associated with increased
15 pathogenicity. The IL-1 β receptor is an exception: its presence diminishes the host febrile response and enhances host survival in the face of infection (Alcamì *et al.* 1996). We have discovered that MVA lacks functional cytokine receptors for interferon γ , interferon $\alpha\beta$, Tumour Necrosis Factor and CC chemokines, but it does possess the potentially
20 beneficial IL-1 β receptor. MVA is the only known strain of vaccinia to possess this cytokine receptor profile, which theoretically renders it safer and more immunogenic than other poxviruses. Another replication-impaired and safe strain of vaccinia known as NYVAC is fully described in Tartaglia *et al.* (Virology 1992, 188: 217-232).

25 It has long been recognised that live viruses have some attractive features as recombinant vaccine vectors including a high capacity for foreign antigens and fairly good immunogenicity for cellular immune responses (Ellis 1988 new technologies for making vaccines. In: Vaccines. Editors: Plotkin S A and Mortimer E A. W B Saunders,
30 Philadelphia, page 568; Woodrow G C 1977. In: New Generation

Vacciness 2nd Edition. Editors: Levine M M, Woodrow G C, Kaper J B, Cobon G, page 33). This has led to attempts to attenuate the virulence of such live vectors in various ways including reducing their replication capacity (Tartaglia J *et al.* 1992 Virology 188: 217-232). However such a
5 reduction in replication reduces the amount of antigen produced by the virus and thereby would be expected to reduce vaccine immunogenicity. Indeed attenuation of replicating vaccinia strains has previously been shown to lead to some substantial reductions in antibody responses (Lee M S *et al.*, 1992 J Virology 66: 2617-2630). Similarly the non-replicating
10 fowlpox vector was found to be less immunogenic for antibody production and less protective than a replicating wild-type vaccinia strain in a rabies study (Taylor J *et al.* 1991 Vaccine 9: 190-193).

It has now been discovered that non-replicating and replication-impaired strains of poxvirus provide vectors which give an
15 extremely good boosting effect to a primed CTL response. Remarkably, this effect is significantly stronger than a boosting effect by wild type poxviruses. The effect is observed with malarial and other antigens such as viral and tumour antigens, and is protective as shown in mice and non-human primate challenge experiments. Complete rather than partial
20 protection from sporozoite challenge has been observed with the novel immunisation regime.

It is an aim of this invention to identify an effective means of immunising against malaria. It is a further aim of this invention to identify means of immunising against other diseases in which CD8+ T cell
25 responses play a protective role. Such diseases include but are not limited to infection and disease caused by the viruses HIV, herpes simplex, herpes zoster, hepatitis C, hepatitis B, influenza, Epstein-Barr virus, measles, dengue and HTLV-1; by the bacteria *Mycobacterium tuberculosis* and *Listeria* sp.; and by the protozoan parasites *Toxoplasma* and

Trypanosoma; and certain forms of cancer e.g. melanoma, cancer of the breast and cancer of the colon.

We describe here a novel method of immunising that generated very high levels of CD8+ T cells and was found to be capable of inducing unprecedented complete protection against *P. berghei* sporozoite challenge. The same approach was tested in higher primates and found to be highly immunogenic in this species also, and was found to induce partial protection against *P. falciparum* challenge. Induction of protective immune responses has also been demonstrated in two additional mouse models of viral infection and cancer.

We show further than the novel immunisation regime that is described here is also effective in generating strong CD8+ T cell responses against HIV epitopes. Considerable evidence indicates that the generation of such CD8+ T cell responses can be expected to be of value in prophylactic or therapeutic immunisation against this viral infection and disease (Gallimore *et al* 1995; Ada 1996). We demonstrate that strong CD8+T cell responses may be generated against epitopes from both HIV and malaria using an epitope string with sequences from both of these micro-organisms. The success in generating enhanced immunogenicity against both HIV and malaria epitopes, and also against influenza and tumour epitopes, indicates that this novel immunisation regime can be effective generally against many infectious pathogens and also in non-infectious diseases where the generation of a strong CD8+ T cell response may be of value.

A surprising feature of the current invention is the finding of the very high efficacy of non-replicating agents in both priming and particularly in boosting a CD8+ T cell response. In general the immunogenicity of CD8+ T cell induction by live replicating viral vectors has previously been found to be higher than for non-replicating agents or replication-impaired vectors. This is as would be expected from the greater

amount of antigen produced by agents that can replicate in the host. Here however we find that the greatest immunogenicity and protective efficacy is surprisingly observed with non-replicating vectors. The latter have an added advantage for vaccination in that they are in general safer for use in humans than replicating vectors.

The present invention provides in one aspect a kit for generating a protective CD8+ T cell immune response against at least one target antigen, which kit comprises:

- (i) a priming composition comprising a source of one or more CD8+ T cell epitopes of the target antigen, together with a pharmaceutically acceptable carrier; and
- (ii) a boosting composition comprising a source of one or more CD8+ T cell epitopes of the target antigen, including at least one CD8+ T cell epitope which is the same as a CD8+ T cell epitope of the priming composition, wherein the source of CD8+ T cell epitopes is a non-replicating or replication-impaired recombinant poxvirus vector, together with a pharmaceutically acceptable carrier;

with the proviso that if the source of epitopes in (i) is a viral vector, the viral vector in (ii) is derived from a different virus.

In another aspect the invention provides a method for generating a protective CD8+ T cell immune response against at least one target antigen, which method comprises administering at least one dose of component (i), followed by at least one dose of component (ii) of the kit according to the invention.

Preferably, the source of CD8+ T cell epitopes in (i) in the method according to the invention is a non-viral vector or a non-replicating or replication-impaired viral vector, although replicating viral vectors may be used.

Preferably, the source of CD8+ T cell epitopes in (i) is not a poxvirus vector, so that there is minimal cross-reactivity between the primer and the booster.

In one preferred embodiment of the invention, the source of
5 CD8+ T cell epitopes in the priming composition is a nucleic acid, which may be DNA or RNA, in particular a recombinant DNA plasmid. The DNA or RNA may be packaged, for example in a lysosome, or it may be in free form.

In another preferred embodiment of the invention, the source
10 of CD8+ T cell epitopes in the priming composition is a peptide, polypeptide, protein, polyprotein or particle comprising two or more CD8+ T cell epitopes, present in a recombinant string of CD8+ T cell epitopes or in a target antigen. Polyproteins include two or more proteins which may be the same, or preferably different, linked together. Particularly preferred in
15 this embodiment is a recombinant proteinaceous particle such as a Ty virus-like particle (VLP) (Burns *et al.* Molec. Biotechnol. 1994, 1: 137-145).

Preferably, the source of CD8+ T cell epitopes in the boosting composition is a vaccinia virus vector such as MVA or NYVAC. Most preferred is the vaccinia strain modified virus ankara (MVA) or a strain
20 derived therefrom. Alternatives to vaccinia vectors include avipox vectors such as fowlpox or canarypox vectors. Particularly suitable as an avipox vector is a strain of canarypox known as ALVAC (commercially available as Kanapox), and strains derived therefrom.

Poxvirus genomes can carry a large amount of heterologous
25 genetic information. Other requirements for viral vectors for use in vaccines include good immunogenicity and safety. MVA is a replication-impaired vaccinia strain with a good safety record. In most cell types and normal human tissues, MVA does not replicate; limited replication of MVA is observed in a few transformed cell types such as BHK21 cells. It has
30 now been shown, by the results described herein, that recombinant MVA

and other non-replicating or replication-impaired strains are surprisingly and significantly better than conventional recombinant vaccinia vectors at generating a protective CD8+ T cell response, when administered in a boosting composition following priming with a DNA plasmid, a recombinant
5 Ty-VLP or a recombinant adenovirus.

It will be evident that vaccinia virus strains derived from MVA, or independently developed strains having the features of MVA which make MVA particularly suitable for use in a vaccine, will also be suitable for use in the invention.

10 MVA containing an inserted string of epitopes (MVA-HM, which is described in the Examples) has been deposited at the European Collection of Animal Cell Cultures, CAMR, Salisbury, Wiltshire SP4 0JG, UK under accession no. V97060511 on 5 June 1997.

The term "non-replicating" or "replication-impaired" as used
15 herein means not capable of replication to any significant extent in the majority of normal mammalian cells or normal human cells. Viruses which are non-replicating or replication-impaired may have become so naturally (i.e. they may be isolated as such from nature) or artificially e.g. by breeding *in vitro* or by genetic manipulation, for example deletion of a gene
20 which is critical for replication. There will generally be one or a few cell types in which the viruses can be grown, such as CEF cells for MVA.

Replication of a virus is generally measured in two ways:
1) DNA synthesis and 2) viral titre. More precisely, the term "non-replicating or replication-impaired" as used herein and as it applies to
25 poxviruses means viruses which satisfy either or both of the following criteria:

- 1) exhibit a 1 log (10 fold) reduction in DNA synthesis compared to the Copenhagen strain of vaccinia virus in MRC-5 cells (a human cell line);

- 2) exhibit a 2 log reduction in viral titre in HELA cells (a human cell line) compared to the Copenhagen strain of vaccinia virus.

Examples of poxviruses which fall within this definition are
5 MVA, NYVAC and avipox viruses, while a virus which falls outside the definition is the attenuated vaccinia strain M7.

Alternative preferred viral vectors for use in the priming composition according to the invention include a variety of different viruses, genetically disabled so as to be non-replicating or replication-impaired.
10 Such viruses include for example non-replicating adenoviruses such as E1 deletion mutants. Genetic disabling of viruses to produce non-replicating or replication-impaired vectors has been widely described in the literature (e.g. McLean *et al.* 1994).

Other suitable viral vectors for use in the priming composition
15 are vectors based on herpes virus and Venezuelan equine encephalitis virus (VEE) (Davies *et al.* 1996). Suitable bacterial vectors for priming include recombinant BCG and recombinant *Salmonella* and *Salmonella* transformed with plasmid DNA (Darji A *et al.* 1997 Cell 91: 765-775).

Alternative suitable non-viral vectors for use in the priming
20 composition include lipid-tailed peptides known as lipopeptides, peptides fused to carrier proteins such as KLH either as fusion proteins or by chemical linkage, whole antigens with adjuvant, and other similar systems. Adjuvants such as QS21 or SBAS2 (Stoute J A *et al.* 1997 N Engl J Medicine 226: 86-91) may be used with proteins, peptides or nucleic acids
25 to enhance the induction of T cell responses. These systems are sometimes referred to as "immunogens" rather than "vectors", but they are vectors herein in the sense that they carry the relevant CD8+ T cell epitopes.

There is no reason why the priming and boosting
30 compositions should not be identical in that they may both contain the

priming source of CD8+ T cell epitopes as defined in (i) above and the boosting source of CD8+ T cell epitopes as defined in (ii) above. A single formulation which can be used as a primer and as a booster will simplify administration. The important thing is that the primer contains at least the
5 priming source of epitopes as defined in (i) above and the booster contains at least the boosting source of epitopes as defined in (ii) above.

The CD8+ T cell epitopes either present in, or encoded by the priming and boosting compositions, may be provided in a variety of different forms, such as a recombinant string of one or two or more
10 epitopes, or in the context of the native target antigen, or a combination of both of these. CD8+ T cell epitopes have been identified and can be found in the literature, for many different diseases. It is possible to design epitope strings to generate a CD8+ T cell response against any chosen antigen that contains such epitopes. Advantageously, the epitopes in a
15 string of multiple epitopes are linked together without intervening sequences so that unnecessary nucleic acid and/or amino acid material is avoided. In addition to the CD8+ T cell epitopes, it may be preferable to include one or more epitopes recognised by T helper cells, to augment the immune response generated by the epitope string. Particularly suitable T
20 helper cell epitopes are ones which are active in individuals of different HLA types, for example T helper epitopes from tetanus (against which most individuals will already be primed). A useful combination of three T helper epitopes is employed in the examples described herein. It may also be useful to include B cell epitopes for stimulating B cell responses and
25 antibody production.

The priming and boosting compositions described may advantageously comprise an adjuvant. In particular, a priming composition comprising a DNA plasmid vector may also comprise granulocyte macrophage-colony stimulating factor (GM-CSF), or a plasmid

encoding it, to act as an adjuvant; beneficial effects are seen using GM-CSF in polypeptide form.

The compositions described herein may be employed as therapeutic or prophylactic vaccines. Whether prophylactic or therapeutic immunisation is the more appropriate will usually depend upon the nature of the disease. For example, it is anticipated that cancer will be immunised against therapeutically rather than before it has been diagnosed, while anti-malaria vaccines will preferably, though not necessarily be used as a prophylactic.

The compositions according to the invention may be administered via a variety of different routes. Certain routes may be favoured for certain compositions, as resulting in the generation of a more effective response, or as being less likely to induce side effects, or as being easier for administration. The present invention has been shown to be effective with gene gun delivery, either on gold beads or as a powder.

In further aspects, the invention provides:

- a method for generating a protective CD8+ T cell immune response against a pathogen or tumour, which method comprises administering at least one dose of a recombinant DNA plasmid encoding at least one CD8+ T cell epitope or antigen of the pathogen or cancer, followed by at least one dose of a non-replicating or replication-impaired recombinant pox virus encoding the same epitope or antigen;
- a method for generating a protective CD8+ T cell immune response against a pathogen or tumour, which method comprises administering at least one dose of a recombinant protein or particle comprising at least one epitope or antigen of the pathogen or cancer, followed by at least one dose of a recombinant MVA vector encoding the same epitope or antigen;

- the use of a recombinant non-replicating or replication-impaired pox virus vector in the manufacture of a medicament for boosting a CD8+ T cell immune response;
- the use of an MVA vector in the manufacture of a medicament for boosting a CD8+ T cell immune response;
- a medicament for boosting a primed CD8+ T cell response against at least one target antigen or epitope, comprising a source of one or more CD8+ T cell epitopes of the target antigen, wherein the source of CD8+ T cell epitopes is a non-replicating or a replication-impaired recombinant poxvirus vector, together with a pharmaceutically acceptable carrier; and
- the priming and/or boosting compositions described herein, in particulate form suitable for delivery by a gene gun; and methods of immunisation comprising delivering the compositions by means of a gene gun.

Also provided by the invention are: the epitope strings described herein, including epitope strings comprising the amino acid sequences listed in table 1 and table 2; recombinant DNA plasmids encoding the epitope strings; recombinant Ty-VLPs comprising the epitope strings; a recombinant DNA plasmid or non-replicating or replication impaired recombinant pox virus encoding the *P. falciparum* antigen TRAP; and a recombinant polypeptide comprising a whole or substantially whole protein antigen such as TRAP and a string of two or more epitopes in sequence such as CTL epitopes from malaria.

25

Example Formulations and Immunisation Protocols

Formulation 1

Priming Composition: DNA plasmid 1 mg/ml in PBS

Boosting Composition: Recombinant MVA, 10^8 ffu in PBS

30

Protocol: Administer two doses of 1 mg of priming composition, i.m., at 0 and 3 weeks followed by two doses of booster intradermally at 6 and 9 weeks.

5 **Formulation 2**

Priming Composition: Ty-VLP 500 μ g in PBS

Boosting Composition: MVA, 10⁸ ffu in PBS

Protocol: Administer two doses of priming composition, i.m., at 0 and 3
10 weeks, then 2 doses of booster at 6 and 9 weeks. For tumour treatment, MVA is given i.v. as one of most effective routes.

Formulation 3

Priming Composition: Protein 500 μ g + adjuvant (QS-21)

15 Boosting Composition: Recombinant MVA, 10⁸ ffu in PBS

Protocol: Administer two doses of priming composition at 0 and 3 weeks and 2 doses of booster i.d. at 6 and 9 weeks.

20 **Formulation 4**

Priming Composition: Adenovirus vector, 10⁹ pfu in PBS

Boosting Composition: Recombinant MVA, 10⁸ ffu in PBS

Protocol: Administer one or two doses of priming composition
25 intradermally at 0 and 3 weeks and two doses of booster i.d. at 6 and 9 weeks.

The above doses and protocols may be varied to optimise protection.

Doses may be given between for example, 1 to 8 weeks apart rather than
30 2 weeks apart.

The invention will now be further described in the examples which follow.

EXAMPLES

5 EXAMPLE 1

Materials and Methods

Generation of the epitope strings.

The malaria epitope string was made up of a series of cassettes each encoding three epitopes as shown in Table 1, with
 10 restriction enzyme sites at each end of the cassette. Each cassette was constructed from four synthetic oligonucleotides which were annealed together, ligated into a cloning vector and then sequenced to check that no errors had been introduced. Individual cassettes were then joined together as required. The *Bam*HI site at the 3' end of cassette C was fused to the
 15 *Bgl*II site at the 5' end of cassette A, destroying both restriction enzyme sites and encoding a two amino acid spacer (GS) between the two cassettes. Cassettes B, D and H were then joined to the string in the same manner. A longer string containing CABDHFE was also constructed in the same way.

20

Table 1. CTL epitopes of the malaria (M) string

Cassette	Epitope	Amino acid Sequence	DNA sequence	Type	HLA restriction
A	Ls8	KPNDKSLY	AAGCCGAACGACAAGTCCTTGAT	CTL	B35
	Cp26	KPKDEL DY	AAACCTAAGGACGAATTGGACTAC	CTL	B35
	Ls6	KPIVQYDNF	AAGCCAATCGTTCAATACGACAATT C	CTL	B53
B	Tr42/43	ASKNKEKALII	GCCTCCAAGAACAAGGAAAAGGCTTTGATCA TC	CTL	B8
	Tr39	GIAGGLALL	GGTATCGCTGGTGGTTTGGCCTTGTTG	CTL	A2.1
	Cp6	MNPNDPNRN V	ATGAACCCTAATGACCCAAACAGAAACGTC	CTL	B7

C	St8	MINAYLDKL	ATGATCAACGCCTACTTGGACAAGTTG	CTL	A2.2
	Ls50	ISKYEDEI	ATCTCCAAGTACGAAGACGAAATC	CTL	B17
	Pb9	SYIPSAEKI	TCCTACATCCCATCTGCCGAAAAGATC	CTL	mouse H2-K ^d
D	Tr26	HLGNVKYLV	CAC TTGGGTAACGTTAAGTACTTGGTT	CTL	A2.1
	Ls53	KSLYDEHI	AAGTCTTTGTACGATGAACACATC	CTL	B58
	Tr29	LLMDCSGSI	TTATTGATGGACTGTTCTGGTTCTATT	CTL	A2.2
E	NANP	NANPNANPN ANPNANP	AACGCTAATCCAAACGCAAATCCGAACGCCA ATCCTAACGCGAATCCC	B cell	
	TRAP AM	DEWSPCSVT CGKGTRSRK RE	GACGAATGGTCTCCATGTTCTGTCACTTGTG GTAAGGGTACTCGCTCTAGAAAGAGAGAA	Heparin binding motif	
F	Cp39	YLNKIQNSL	TACTTGAACAAAATTCAAACCTCTTTG	CTL	A2.1
	La72	MEKLKELEK	ATGGAAAAGTTGAAAGAATTGGAAAAG	CTL	B8
	ex23	ATSVLAGL	GCTACTTCTGTCTTGGCTGGTTTG	CTL	B58
H	CSP	DPNANPNVD PNANPNV	GACCCAAACGCTAACCCAAACGTTGACCCA AACGCCAACCCAAACGTC	T helper	Universal epitopes
	BCG	QVHFQPLPP AVVKL	CAAGTTCACTTCCAACCAATTGCCTCCGGCCG TTGTCAAGTTG	T helper	
	TT	QFIKANSKFI GITE	CAATTCATCAAGGCCAACTCTAAGTTCATCG GTATCACCGAA	T helper	

Table 1 Sequences included in the malaria epitope string. Each cassette consists of the epitopes shown above, in the order shown, with no additional sequence between epitopes within a cassette. A BglII site was added at the 5' end and a BamHI site at the 3' end, such that between cassettes in an epitope string the BamHI/BglII junction encodes GS. All epitopes are from *P. falciparum* antigens except for pb9 (*P. berghei*), BCG (*M. tuberculosis*) and TT (Tetanus). The amino acid and DNA sequences shown in the table have SEQ ID NOS. 1 to 40 in the order in which they appear.

Figure 1 shows the construct used to express Ty-VLP with the malaria epitope cassette CABDHFE. CTL epitopes are from *P. falciparum* STARP (sporozoite threonine- and asparagine-rich protein) (st), LSA-1 (liver stage antigen 1) (1s), CSP (circumsporozoite protein) (cp),

TRAP (thrombospondin-related adhesive protein) (tr), LSA-3 (liver stage antigen 3) (la) and Exp-1 (exported protein 1) (ex). Helper epitopes are from the *P. falciparum* CS protein, the *M. tuberculosis* 38Kd antigen and Tetanus Toxoid. NANP is the antibody epitope from CS and AM is the
 5 adhesion motif from *P. falciparum* TRAP (Muller *et al* 1993). The length of the complete string is 229 amino acids as shown in the table 1 legend, with the amino acid sequence:-

MINAYLDKLISKYEDEISYIPSAEKIGSKPNDKSLYKPKDEL DYKPIVQYDN
 FGSASKNKEKALIIGIAGGLALLMNPNDPNRNVGSHLGNVKYL VKSLYDE
 10 HILLMDCSGSIGSDPNANPNVDPNANPNVQVHFQPLPPAVVKLQFIKANS
 KFIGITEGSYLNKIQNLSMEKLKELEKATSVLAGLGSNANPNANPNANPNA
 NPDEWSPCSVTGCGKGRSRKREGSGK [SEQ ID NO: 41].

The HIV epitope string was also synthesised by annealing oligonucleotides. Finally the HIV and malaria epitope strings were fused
 15 by joining the *Bam*HI site at the 3' end of the HIV epitopes to the *Bgl*II site at the 5' end of cassettes CAB to form the HM string (Table 2)

Table 2 CTL epitopes of the HIV/SIV epitope string

Epitope	Restriction	Origin
YLKDQQLL	A24, B8	HIV-1 gp41
ERYLKDQQL	B14	HIV-1 gp41
EITPIGLAP	Mamu-B*01	SIV env
PPIPVGEIY	B35	HIV-1 p24
GEIYKRWII	B8	HIV-1 p24
KRWIILGLNK	B*2705	HIV-1 p24
IILGLNKIVR	A33	HIV-1 p24
LGLNKIVRMY	Bw62	HIV-1 p24
YNLTMKCR	Mamu-A*02	SIV env
RGPGRAFTI	A2, H-2Dd	HIV-1 gp120
GRAFTIGK	B*2705	HIV-1 gp120
TPYDINQML	B53	HIV-2 gag
CTPYDINQM	Mamu-A*01	SIV gag

RPQVPLRPMTY	B51	HIV-1 nef
QVPLRPMTYK	A*0301, A11	HIV-1 nef
VPLRPMTY	B35	HIV-1 nef
AVDLSHFLK	A11	HIV-1 nef
DLSHFLKEK	A*0301	HIV-1 nef
FLKEKGGL	B8	HIV-1 nef
ILKEPVHGV	A*0201	HIV-1 pol
ILKEPVHGVY	Bw62	HIV-1 pol
HPDIVIYQY	B35	HIV-1 pol
VIYQYMDDL	A*0201	HIV-1 pol

Table 2 Sequences of epitopes from HIV or SIV contained in the H
 5 epitope string and assembled as shown in figure 2. The amino acids in the
 table have SEQ ID NOS: 42 to 64 in the order in which they appear.

Figure 2 shows a schematic outline of the H, M and HM
 proteins. The bar patterns on the schematic representations of the
 polypeptide proteins indicate the origin of the sequences (see tables 1 and
 10 2). The positions of individual epitopes and their MHC restrictions are
 depicted above and below the proteins. Pb is the only epitope derived from
 the protein of *P. berghei*. All other epitopes in the M protein originate from
 proteins of *P. falciparum*: cs – circumsporozoite protein, st – STARP, ls –
 LSA-1 and tr – TRAP. BCG – 38 kDa protein of *M. tuberculosis*; TT –
 15 tetanus toxin.

For the anti-tumour vaccine an epitope string containing CTL
 epitopes was generated, similar to the malaria and HIV epitope string. In
 this tumour epitope string published murine CTL epitopes were fused
 together to create the tumour epitope string with the amino acid sequence:
 20 **MLPYLGWLVF-AQHPNAELL-KHYLFRNL-SPSYVYHQF-IPNPLLGLD**
 [SEQ ID NO: 65]. CTL epitopes shown here were fused together. The first
 amino acid methionine was introduced to initiate translation.

Ty virus-like particles (VLPs).

The epitope string containing cassette CABDH was introduced into a yeast expression vector to make a C-terminal in-frame fusion to the TyA protein. When TyA or TyA fusion proteins are expressed in yeast from this vector, the protein spontaneously forms virus like particles which can be purified from the cytoplasm of the yeast by sucrose gradient centrifugation. Recombinant Ty-VLPs were prepared in this manner and dialysed against PBS to remove the sucrose before injection (c.f. Layton *et al.* 1996).

Adenoviruses

Replication-defective recombinant Adenovirus with a deletion of the E1 genes was used in this study (McGrory *et al.* 1988). The Adenovirus expressed *E. coli* β -galactosidase under the control of a CMV IE promoter. For immunisations, 10^7 pfu of virus were administered intradermally into the ear lobe.

Peptides

Peptides were purchased from Research Genetics (USA), dissolved at 10 mg/ml in DMSO (Sigma) and further diluted in PBS to 1 mg/ml. Peptides comprising CTL epitopes that were used in the experiments described herein are listed in table 3

Table 3 Sequence of CTL peptide epitopes

sequence	Antigen	MHC restriction
LPYLGWLVF	P1A tumour antigen	L ^d
SYIPSAEKI	<i>P. berghei</i> CSP	K ^d
RGPGRAFTI	HIV gag	D ^d

TPHPARIGL	<i>E. coli</i> β -galactosidase	L ^d
TYQRTRALV	Influenza A virus NP	K ^d
SDYEGRLI	Influenza A virus NP	K ^k
ASNENMETM	Influenza A virus NP	D ^b
INVAFNRFL	<i>P. falciparum</i> TRAP	K ^b

The amino acid sequences in Table 3 have SEQ ID NOS: 66 to 73, in the order in which they appear in the Table.

5 *Plasmid DNA constructs*

A number of different vectors were used for constructing DNA vaccines. Plasmid pTH contains the CMV IE promoter with intron A, followed by a polylinker to allow the introduction of antigen coding sequences and the bovine growth hormone transcription termination sequence. The plasmid carries the ampicillin resistance gene and is capable of replication in *E. coli* but not mammalian cells. This was used to make DNA vaccines expressing each of the following antigens: *P. berghei* TRAP, *P. berghei* CS, *P. falciparum* TRAP, *P. falciparum* LSA-1 (278 amino acids of the C terminus only), the epitope string containing cassettes CABDH and the HM epitope string (HIV epitopes followed by cassettes CAB). Plasmid pSG2 is similar to pTH except for the antibiotic resistance gene. In pSG2 the ampicillin resistance gene of pTH has been replaced by a kanamycin resistance gene. pSG2 was used to make DNA vaccines expressing the following antigens: *P. berghei* PbCSP, a mouse tumour epitope string, the epitope string containing cassettes CABDH and the HM epitope string. Plasmid V1J-NP expresses influenza nucleoprotein under the control of a CMV IE promoter. Plasmids CMV-TRAP and CMV-LSA-1 are similar to pTH.TRAP and pTH. LSA-1 but do not contain intron A of the CMV promoter. Plasmids RSV.TRAP and RSV.LSA-1 contain the RSV promoter, SV40 transcription termination sequence and are tetracycline

resistant. For induction of β -galactosidase-specific CTL plasmid pcDNA3/His/LacZ (Invitrogen) was used. All DNA vaccines were prepared from *E. coli* strain DH5 α using Qiagen plasmid purification columns.

5 *Generation of recombinant vaccinia viruses*

Recombinant MVAs were made by first cloning the antigen sequence into a shuttle vector with a viral promoter such as the plasmid pSC11 (Chakrabarti *et al.* 1985; Morrison *et al.* 1989). *P. berghei* CS and *P. falciparum* TRAP, influenza nucleoprotein and the HM and mouse
10 tumour epitope polyepitope string were each expressed using the P7.5 promoter (Mackett *et al.* 1984), and *P. berghei* TRAP was expressed using the strong synthetic promoter (SSP; Carroll *et al.* 1995). The shuttle vectors, pSC11 or pMCO3 were then used to transform cells infected with wild-type MVA so that viral sequences flanking the promoter, antigen
15 coding sequence and marker gene could recombine with the MVA and produce recombinants. Recombinant viruses express the marker gene (β glucuronidase or β galactosidase) allowing identification of plaques containing recombinant virus. Recombinants were repeatedly plaque purified before use in immunisations. The recombinant NYVAC-PbCSP
20 vaccinia was previously described (Lanar *et al.* 1996). The wild type or Western Reserve (WR) strain of recombinant vaccinia encoding PbCSP was described previously (Satchidanandam *et al.* 1991).

Cells and culture medium

25 Murine cells and Epstein-Barr virus transformed chimpanzee and macaque B cells (BCL) were cultured in RPMI supplemented with 10% heat inactivated fetal calf serum (FCS). Splenocytes were restimulated with the peptides indicated (final concentration 1 μ g/ml) in MEM medium with 10% FCS, 2mM glutamine, 50U/ml penicillin, 50 μ M 2-
30 mercaptoethanol and 10mM Hepes pH7.2 (Gibco, UK).

Animals

Mice of the strains indicated, 6-8 weeks old were purchased from Harlan Olac (Shaws Farm, Blackthorn, UK). Chimpanzees H1 and
5 H2 were studied at the Biomedical Primate Research Centre at Rijswijk, The Netherlands. Macaques were studied at the University of Oxford.

Immunisations

Plasmid DNA immunisations of mice were performed by
10 intramuscular immunisation of the DNA into the musculus tibialis under anaesthesia. Mouse muscle was sometimes pre-treated with 50 µl of 1mM cardiotoxin (Latoxan, France) 5-9 days prior to immunisation as described by Davis *et al* (1993), but the presence or absence of such pre-treatment was not found to have any significant effect on immunogenicity or
15 protective efficacy. MVA immunisation of mice was performed by either intramuscular (i.m.), intravenous (into the lateral tail vein) (i.v.), intradermal (i.d.), intraperitoneal (i.p.) or subcutaneous (s.c.) immunisation. Plasmid DNA and MVA immunisation of the chimpanzees H1 and H2 was performed under anaesthesia by intramuscular immunisation of leg
20 muscles. For these chimpanzee immunisations the plasmid DNA was co-administered with 15 micrograms of human GM-CSF as an adjuvant. Recombinant MVA administration to the chimpanzees was by intramuscular immunisation under veterinary supervision. Recombinant human GM-CSF was purchased from Sandoz (Camberley, UK). For
25 plasmid DNA immunisations using a gene gun, DNA was precipitated onto gold particles. For intradermal delivery, two different types of gene guns were used, the Acell and the Oxford Bioscience device (PowderJect Pharmaceuticals, Oxford, UK).

ELISPOT assays

CD8+ T cells were quantified in the spleens of immunised mice without *in vitro* restimulation using the peptide epitopes indicated and the ELISPOT assay as described by Miyahara *et al* (1993). Briefly, 96-well nitrocellulose plates (Miliscreen MAHA, Millipore, Bedford UK) were coated with 15 µg/ml of the anti-mouse interferon-γ monoclonal antibody R4 (EACC) in 50 µl of phosphate-buffered saline (PBS). After overnight incubation at 4°C the wells were washed once with PBS and blocked for 1 hour at room temperature with 100 µl RPMI with 10% FCS. Splenocytes from immunised mice were resuspended to 1 x 10⁷ cells/ml and placed in duplicate in the antibody coated wells and serially diluted. Peptide was added to each well to a final concentration of 1 µg/ml. Additional wells without peptide were used as a control for peptide-dependence of interferon-γ secretion. After incubation at 37°C in 5%CO₂ for 12-18 hours the plates were washed 6 times with PBS and water. The wells were then incubated for 3 hours at room temperature with a solution of 1 µg/ml of biotinylated anti-mouse interferon-γ monoclonal antibody XMG1.2 (Pharmingen, CA, USA) in PBS. After further washes with PBS, 50 µl of a 1 µg/ml solution of streptavidin-alkaline-phosphatase polymer (Sigma) was added for 2 hours at room temperature. The spots were developed by adding 50 µl of an alkaline phosphatase conjugate substrate solution (Biorad, Hercules, CA, USA). After the appearance of spots the reaction was stopped by washing with water. The number of spots was determined with the aid of a stereomicroscope.

25

ELISPOT assays on the chimpanzee peripheral blood lymphocytes were performed using a very similar method employing the assay and reagents developed to detect human CD8 T cells (Mabtech, Stockholm).

30

CTL assays

CTL assays were performed using chromium labelled target cells as indicated and cultured mouse spleen cells as effector cells as described by Allsopp *et al.* (1996). CTL assays using chimpanzee or
5 macaque cells were performed as described for the detection of human CTL by Hill *et al.* (1992) using EBV-transformed autologous chimpanzee chimpanzee or macaque B cell lines as target cells.

P. berghei challenge

10 Mice were challenged with 2000 (BALB/c) or 200 (C57BL/6) sporozoites of the *P. berghei* ANKA strain in 200 µl RPMI by intravenous inoculation as described (Lanar *et al.* 1996). These sporozoites were dissected from the salivary glands of *Anopheles stephensi* mosquitoes maintained at 18°C for 20-25 days after feeding on infected mice. Blood-
15 stage malaria infection, indicating a failure of the immunisation, was detected by observing the appearance of ring forms of *P. berghei* in Giemsa-stained blood smears taken at 5-12 days post-challenge.

P. falciparum challenge

20 The chimpanzees were challenged with 20,000 *P. falciparum* sporozoites of the NF54 strain dissected from the salivary glands of *Anopheles gambiae* mosquitoes, by intravenous inoculation under anaesthesia. Blood samples from these chimpanzees were examined daily from day 5 after challenge by microscopy and parasite culture, in
25 order to detect the appearance of low levels of *P. falciparum* parasites in the peripheral blood.

P815 tumour challenges

Mice were challenged with 1×10^5 P815 cells in 200 µl of
30 PBS by intravenous inoculation. Animals were monitored for survival.

Influenza virus challenges

Mice were challenged with 100 haemagglutinating units (HA) of influenza virus A/PR/8/34 by intranasal inoculation. Following challenge
 5 the animals were weighed daily and monitored for survival.

Determining peptide specific CTL using tetramers

Tetrameric complexes consisting of Mamu-A*01-heavy chain and β_2 -microglobulin were made as described by Ogg *et al* (1998). DNA
 10 coding for the leaderless extracellular portion of the Mamu-A*01 MHC class I heavy chain was PCR-amplified from cDNA using 5'primer MamuNdeI: 5'-CCT GAC TCA GAC CAT ATG GGC TCT CAC TCC ATG [SEQ ID NO: 74] and 3' primer: 5'-GTG ATA AGC TTA ACG ATG ATT CCA CAC CAT TTT CTG TGC ATC CAG AAT ATG ATG CAG GGA TCC
 15 CTC CCA TCT CAG GGT GAG GGG C [SEQ ID NO: 75]. The former primer contained a NdeI restriction site, the latter included a HindIII site and encoded for the bioinylation enzyme BirA substrate peptide. PCR products were digested with NdeI and HindIII and ligated into the same sites of the polylinker of bacterial expression vector pGMT7. The rhesus
 20 monkey gene encoding a leaderless β_2 -microglobulin was PCR amplified from a cDNA clone using primers B2MBACK: 5'-TCA GAC CAT ATG TCT CGC TCC GTG GCC [SEQ ID NO: 76] and B2MFOR: 5'-TCA GAC AAG CTT TTA CAT GTC TCG ATC CCA C [SEQ ID NO: 77] and likewise cloned into the NdeI and HindIII sites of pGMT7. Both chains were
 25 expressed in *E. coli* strain BL-21, purified from inclusion bodies, refolded in the presence of peptide CTPYDINQM [SEQ ID NO: 54], biotinylated using the BirA enzyme (Avidity) and purified with FPLC and monoQ ion exchange columns. The amount of biotinylated refolded MHC-peptide
 30 complexes was estimated in an ELISA assay, whereby monomeric complexes were first captured by conformation sensitive monoclonal

antibody W6/32 and detected by alkaline phosphatase (AP) –conjugated streptavidin (Sigma) followed by colorimetric substrate for AP. The formation of tetrameric complexes was induced by addition of phycoerythrin (PE)-conjugated streptavidin (ExtrAvidin; Sigma) to the
5 refolded biotinylated monomers at a molar ratio of MHC-peptide : PE-streptavidin of 4 : 1. The complexes were stored in the dark at 4°C. These tetramers were used to analyse the frequency of Mamu-A*01/gag-specific CD8+ T cells in peripheral blood lymphocytes (PBL) of immunised macaques.

10

EXAMPLE 2

Immunogenicity Studies in Mice

Previous studies of the induction of CTL against epitopes in the circumsporozoite (CS) protein of *Plasmodium berghei* and *Plasmodium*
15 *yoelii* have shown variable levels of CTL induction with different delivery systems. Partial protection has been reported with plasmid DNA (Sedegah *et al.* 1994), influenza virus boosted by replicating vaccinia virus (Li *et al.* 1991), adenovirus (Rodrigues *et al.* 1997) and particle delivery systems (Schodel *et al.* 1994). Immunisation of mice intramuscularly with 50
20 micrograms of a plasmid encoding the CS protein produced moderate levels of CD8+ cells and CTL activity in the spleens of these mice after a single injection (Figures 3, 4).

For comparison groups of BALB/c mice (n = 5) were injected intravenously with 10⁶ ffu/pfu of recombinant vaccinia viruses of different
25 strains (WR, NYVAC and MVA) all expressing *P. berghei* CSP. The frequencies of peptide-specific CD8+ T cells were measured 10 days later in an ELISPOT assay. MVA.PbCSP induced 181 +/- 48, NYVAC 221 +/- 27 and WR 94 +/- 19 (mean +/- standard deviation) peptide-specific CD8+ T cells per million splenocytes. These results show that surprisingly
30 replication-impaired vaccinia viruses are superior to replicating strains in

priming a CD8+ T cell response. We then attempted to boost these moderate CD8+ T cell responses induced by priming with either plasmid DNA or MVA using homologous or heterologous vectors. A low level of CD8+ T cells was observed after two immunisations with CS recombinant DNA vaccine alone, the recombinant MVA vaccine alone or the recombinant MVA followed by recombinant DNA (Figure 3). A very much higher level of CD8+ T cells was observed by boosting the DNA-primed immune response with recombinant MVA. In a second experiment using ten mice per group the enhanced immunogenicity of the DNA/MVA sequence was confirmed: DNA/MVA 856 +/- 201; MVA/DNA 168 +/- 72; MVA/MVA 345 +/- 90; DNA/DNA 92 +/- 46. Therefore the sequence of a first immunisation with a recombinant plasmid encoding the CS protein followed by a second immunisation with the recombinant MVA virus yielded the highest levels of CD8+ T lymphocyte response after immunisation.

Figure 3 shows malaria CD8 T cell ELISPOT data following different immunisation regimes. Results are shown as the number of peptide-specific T cells per million splenocytes. Mice were immunised either with the PbCSP-plasmid DNA or the PbCSP-MVA virus or combinations of the two as shown on the X axis, at two week intervals and the number of splenocytes specific for the pb9 malaria epitope assayed two weeks after the last immunisation. Each point represents the number of spot-forming cells (SFCs) measured in an individual mouse. The highest level of CD8+ T cells was induced by priming with the plasmid DNA and boosting with the recombinant MVA virus. This was more immunogenic than the reverse order of immunisation (MVA/DNA), two DNA immunisations (DNA/DNA) or two MVA immunisations (MVA/MVA). It was also more immunogenic than the DNA and MVA immunisations given simultaneously (DNA + MVA 2w), than one DNA immunisation (DNA 4w) or one MVA immunisation given at the earlier or later time point (MVA 2w and MVA 4w).

Figure 4 shows that malaria CD8 T cell ELISPOT and CTL levels are substantially boosted by a recombinant MVA immunisation following priming with a plasmid DNA encoding the same antigen. **A and C.** CD8+ T cell responses were measured in BALB/c mice using the γ -interferon ELISPOT assay on fresh splenocytes incubated for 18 h with the 5 K^d restricted peptide SYIPSAEKI [SEQ ID NO: 67] from *P. berghei* CSP and the L^d restricted peptide TPHPARIGL [SEQ ID NO: 69] from *E. coli* β -galactosidase. Note that the ELISPOT counts are presented on a logarithmic scale. **B and D.** Splenocytes from the same mice were also 10 assayed in conventional ^{51}Cr -release assays at an effector: target ration of 100:1 after 6 days of *in vitro* restimulation with the same peptides (1 $\mu\text{g/ml}$).

The mice were immunised with plasmid DNA expressing either *P. berghei* CSP and TRAP, *PbCSP* alone, the malaria epitope 15 cassette including the *P. berghei* CTL epitope (labelled pTH.M), or β -galactosidase. ELISPOT and CTL levels measured in mice 23 days after one DNA immunisation are shown in A and B respectively. The same assays were performed with animals that received additionally 1×10^7 ffu of recombinant MVA expressing the same antigen(s) two weeks after the 20 primary immunisation. The ELISPOT and CTL levels in these animals are shown in C and D respectively. Each bar represents data from an individual animal.

Studies were also undertaken of the immunogenicity of the epitope string HM comprising both HIV and malaria epitopes in tandem. 25 Using this epitope string again the highest levels of CD8+ T cells and CTL were generated in the spleen when using an immunisation with DNA vaccine followed by an immunisation with a recombinant MVA vaccine (Table 4, Figure 5).

Table 4 Immunogenicity of various DNA/MVA combinations as determined by ELISPOT assays

	Immunisation 1	Immunisation 2	HIV epitope	Malaria epitope
5	DNA-HM	DNA-HM	56 ± 26	4 ± 4
	MVA-HM	MVA-HM	786 ± 334	238 ± 106
10	MVA-HM	DNA-HM	306 ± 78	58 ± 18
	DNA-HM	MVA-HM	1000 ± 487	748 ± 446
	None	DNA-HM	70 ± 60	100 ± 10
15	None	MVA-HM	422 ± 128	212 ± 94

Table 4 shows the results of ELISPOT assays performed to measure the levels of specific CD8+ T cells to HIV and malaria epitopes following different immunisation regimes of plasmid DNA and MVA as indicated. The numbers are spot-forming cells per million splenocytes. The HM epitope string is illustrated in figure 2. BALB/c mice were used in all cases. The malaria epitope was pb9 as in figures 2 and 3. The HIV epitope was RGPGRFVFI [SEQ ID NO: 51]. The immunisation doses were 50 µg of plasmid DNA or 10⁷ focus-forming units (ffu) of recombinant MVA. All immunisations were intramuscular. The interval between immunisations 1 and 2 was from 14-21 days in all cases.

Figure 5 shows the CTL responses induced in BALB/c mice to malaria and HIV epitopes by various immunisation regimes employing plasmid DNA and recombinant MVA. Mice were immunised intramuscularly as described in the legend to table 3 and in methods. High levels of CTL (>30% specific lysis at effector/target ration of 25:1) were observed to both the malaria and HIV epitopes only after priming with

plasmid DNA and boosting with the recombinant MVA. The antigen used in this experiment is the HIV-malaria epitope string. The recombinant MVA is denoted MVA.HM and the plasmid DNA expressing this epitope string is denoted pTH.HM. Levels of specific lysis at various effector to target ratios are shown. These were determined after 5 days *in vitro* restimulation of splenocytes with the two peptides pb9 and RGPGRFVTI [SEQ ID NO: 51].

Comparison of numerous delivery systems for the induction of CTL was reported by Allsopp *et al.* (1996). Recombinant Ty-virus like particles (Ty-VLPs) and lipid-tailed malaria peptides both gave good CTL induction but Ty-VLPs were better in that they required only a single immunising dose for good CTL induction. However, as shown here even two doses of Ty particles fail to induce significant protection against sporozoite challenge (Table 7, line 1). Immunisation with a recombinant modified vaccinia Ankara virus encoding the circumsporozoite protein of *P. berghei* also generates good levels of CTL. However, a much higher level of CD8+ T cell response is achieved by a first immunisation with the Ty-VLP followed by a second immunisation with the MVA CS vaccine (Table 5).

20

Table 5 Immunogenicity of various Ty-VLP/MVA combinations as determined by ELISPOT and CTL assays

Immunisation 1	Immunisation 2	ELISPOT No	%Specific Lysis
Ty-CABDH	Ty- CABDH	75	15
MVA.PbCSP	MVA.PbCSP	38	35
Ty-CABDH	MVA.PbCSP	225	42
Ty- CABDH	MVA.HM	1930	nd

30

Table 5 Results of ELISPOT and CTL assays performed to measure the levels of specific CD8+ T cells to the malaria epitope pb9 following different immunisation regimes of Ty-VLPs and recombinant MVA virus as indicated. The CTL and ELISPOT data are from different experiments.

- 5 The ELISPOT levels (spots per million splenocytes) are measured on un-restimulated cells and the CTL activity, indicated as specific lysis at an effector to target ratio of 40:1, on cells restimulated with pb9 peptide *in vitro* for 5-7 days. Both represent mean levels of three mice. BALB/c mice were used in all cases. The immunisation doses were 50 µg of Ty-VLP or
- 10 10⁷ ffu (foci forming units) of recombinant MVA. All immunisations were intramuscular. The interval between immunisations 1 and 2 was from 14-21 days. MVA.HM includes cassettes CAB.

Priming of an immune response with DNA delivered by a gene gun

15 ***and boosting with recombinant MVA***

Immunogenicity and challenge.

The use of a gene gun to deliver plasmid DNA intradermally and thereby prime an immune response that could be boosted with recombinant MVA was investigated. Groups of BALB/c mice were

20 immunised with the following regimen:

- I) Three gene gun immunisations with pTH.PbCSP (4 mg per immunisation) at two week intervals
- II) Two gene gun immunisations followed by MVA i.v. two weeks later
- 25 III) One intramuscular DNA immunisation followed by MVA i.v. two weeks later.

The immunogenicity of the three immunisation regimens was analysed using ELISPOT assays. The highest frequency of specific T cells was observed with two gene gun immunisations followed by an MVA i.v.

boost and the intramuscular DNA injection followed an MVA i.v. boost (Figure 6).

Figure 6 shows the results of ELISPOT assays performed to measure the levels of specific CD8⁺ T cells to the malaria epitope pb9 following different immunisation regimes. Groups of BALB/c mice (n= 3) were immunised as indicated (g.g. = gene gun). The time between all immunisations was 14 days. ELISPOT assays were done two weeks after the last immunisation.

10 ***CTL induction to the same antigen in different mouse strains***

To address the question whether the boosting effect described above in BALB/c mice with two CTL epitopes SYIPSAEKI [SEQ ID NO: 67] derived from *P. berghei* CSP and RGPGRAFVTI [SEQ ID NO: 68] derived from HIV) is a universal phenomenon, two sets of experiments were carried out. CTL responses to the influenza nucleoprotein were studied in five inbred mouse strains. In a first experiment three published murine CTL epitopes derived from the influenza nucleoprotein were studied (see Table 3). Mice of three different H-2 haplotypes, BALB/c and DBA/2 (H-2^d), C57BL/6 and 129 (H-2^b); CBA/J (H-2^k), were used. One set of animals was immunised twice at two week intervals with the plasmid V1J-NP encoding the influenza nucleoprotein. Another set of identical animals was primed with V1J-NP and two weeks later boosted intravenously with 10⁶ ffu of MVA.NP, expressing influenza virus NP. The levels of CTL in individual mice were determined in a ⁵¹Cr-release assay with peptide re-stimulated splenocytes. As shown in Figure 7, the DNA priming/MVA boosting immunisation regimen induced higher levels of lysis in all the mouse strains analysed and is superior to two DNA injections.

Figure 7 shows the CTL responses against influenza NP in different mouse strains. Mice of different strains were immunised twice two weeks apart with a DNA vaccine V1J-NP encoding for the influenza

nucleoprotein (open circles) or primed with the same DNA vaccine and two weeks later boosted with recombinant MVA expressing influenza virus nucleoprotein (closed circles). Two weeks after the last immunisation splenocytes were restimulated in vitro with the respective peptides (Table
 5 3). The CTL activity was determined in a standard ^{51}Cr -release assay with MHC class I-matched target cells.

CTL induction to different antigens in different mouse strains

The effect of MVA boosting on plasmid DNA-primed immune
 10 responses was further investigated using different antigens and different inbred mouse strains. Mice of different strains were immunised with different antigens using two DNA immunisations and compared with DNA/MVA immunisations. The antigens used were *E. coli* -galactosidase, the malaria/HIV epitope string, a murine tumour epitope string and *P.*
 15 *falciparum* TRAP. Compared with two DNA immunisations the DNA-priming/MVA-boosting regimen induced higher levels of CTL in all the different mouse strains and antigen combinations tested (Figure 8).

Figure 8 shows CTL responses against different antigens induced in different inbred mouse strains. Mice were immunised with two
 20 DNA vaccine immunisations two weeks apart (open circles) or primed with a DNA vaccine and two weeks later boosted with a recombinant MVA expressing the same antigen (closed circles). The strains and antigens were: C57BL/6; *P. falciparum* TRAP in A. DBA/2; *E. coli* β -galactosidase in B. BALB/c; HM epitope string CTL activity against malaria peptide (pb9)
 25 in C. DBA/2; HM epitope string CTL activity against pb9 in D. BALB/c; HM epitope string CTL activity against HIV peptide in E. DBA/2; HM epitope string CTL activity against HIV peptide in F. BALB/c; tumour epitope string CTL activity against P1A-derived peptide in G. DBA/2; tumour epitope string CTL activity against P1A-derived peptide in H. Sequences of peptide
 30 epitopes are shown in table 3. Each curve shows the data for an individual mouse.

Sporozoites can efficiently prime an immune response that is boostable by MVA

Humans living in malaria endemic areas are continuously exposed to sporozoite inoculations. Malaria-specific CTL are found in these naturally exposed individuals at low levels. To address the question whether low levels of sporozoite induced CTL responses can be boosted by MVA, BALB/c mice were immunised with irradiated (to prevent malaria infection) *P. berghei* sporozoites and boosted with MVA. Two weeks after the last immunisation splenocytes were re-stimulated and tested for lytic activity. Two injections with 50 or 300 + 500 sporozoites induced very low or undetectable levels of lysis. Boosting with MVA induced high levels of peptide specific CTL. MVA alone induced only moderate levels of lysis (Figure 9).

Figure 9 shows sporozoite-primed CTL responses are substantially boosted by MVA. Mice were immunised with two low doses (50 + 50) of irradiated sporozoites in A, two high doses (300 + 500) of sporozoites in B; mice were boosted with MVA.PbCSP following low-dose sporozoite priming in D; high dose sporozoite priming in E. CTL responses following immunisation with MVA.PbCSP are shown in C.

Recombinant adenoviruses as priming agent

The prime-boost immunisation regimen has been exemplified using plasmid DNA and recombinant Ty-VLP as priming agent. Here an example using non-replicating adenoviruses as the priming agent is provided. Replication-deficient recombinant Adenovirus expressing *E. coli* β -galactosidase (Adeno-GAL) was used. Groups of BALB/c mice were immunised with plasmid DNA followed by MVA or with Adenovirus followed by MVA. All antigen delivery systems used encoded *E. coli* β -galactosidase. Priming a CTL response with plasmid DNA or Adenovirus and boosting with MVA induces similar levels of CTL (Figure 10).

Figure 10 shows CTL responses primed by plasmid DNA or recombinant Adenovirus and boosted with MVA. Groups of BALB/c mice (n=3) were primed with plasmid DNA A or recombinant Adenovirus expressing β -galactosidase B. Plasmid DNA was administered intramuscularly, MVA intravenously and Adenovirus intradermally. Splenocytes were restimulated with peptide TPHPARIGL [SEQ ID NO: 69] two weeks after the last immunisation. CTL activity was tested with peptide-pulsed P815 cells.

10 ***Immunogenicity of the DNA prime vaccinia boost regimen depends on the replication competence of the strain of vaccinia virus used***

The prime boosting strategy was tested using different strains of recombinant vaccinia viruses to determine whether the different strains with strains differing in their replication competence may differ in their ability to boost a DNA-primed CTL response. Boosting with replication-defective recombinant vaccinia viruses such as MVA and NYVAC resulted in the induction of stronger CTL responses compared to CTL responses following boosting with the same dose of replication competent WR vaccinia virus (Figure 11).

20 Figure 11 shows CTL responses in BALB/c mice primed with plasmid DNA followed by boosting with different recombinant vaccinia viruses. Animals were primed with pTH.PbCSP 50 μ g/mouse i.m. and two weeks later boosted with different strains of recombinant vaccinia viruses (10⁶ pfu per mouse i.v.) expressing PbCSP. The different recombinant vaccinia virus strains were MVA in A; NYVAC in B and WR in C. The superiority of replication-impaired vaccinia strains over replicating strains was found in a further experiment. Groups of BALB/c mice (n = 6) were primed with 50 μ g/animal of pSG2.PbCSP (i.m.) and 10 days later boosted i.v. with 10⁶ ffu/pfu of recombinant MVA, NYVAC and WR expressing
30 PbCSP. The frequencies of peptide-specific CD8⁺ T cells were determined using the ELISPOT assay. The frequencies were: MVA 1103

+/- 438, NYVAC 826 +/- 249 and WR 468 +/- 135. Thus using both CTL assays and ELISPOT assays as a measure of CD8 T cell immunogenicity a surprising substantially greater immunogenicity of the replication-impaired vaccinia strains was observed compared to the replication competent strain.

The use of recombinant canary or fowl pox viruses for boosting CD8+ T cell responses

Recombinant canary pox virus (rCPV) or fowl pox virus (rFVP) are made using shuttle vectors described previously (Taylor *et al.* Virology 1992, 187: 321-328 and Taylor *et al.* Vaccine 1988, 6: 504-508). The strategy for these shuttle vectors is to insert the gene encoding the protein of interest preceded by a vaccinia-specific promoter between two flanking regions comprised of sequences derived from the CPV or FPV genome. These flanking sequences are chosen to avoid insertion into essential viral genes. Recombinant CPV or FPV are generated by *in vivo* recombination in permissive avian cell lines i.e. primary chicken embryo fibroblasts. Any protein sequence of antigens or epitope strings can be expressed using fowl pox or canary pox virus. Recombinant CPV or FPV is characterised for expression of the protein of interest using antigen-specific antibodies or including an antibody epitope into the recombinant gene. Recombinant viruses are grown on primary CEF. An immune response is primed using plasmid DNA as described in Materials and Methods. This plasmid DNA primed immune response is boosted using 10^7 ffu/pfu of rCPV or rFPV inoculated intravenously, intradermally or intramuscularly. CD8+ T cell responses are monitored and challenges are performed as described herein.

EXAMPLE 3

Malaria Challenge Studies in Mice

To assess the protective efficacy of the induced levels of CD8+ T cell response immunised BALB/c or C57BL/6 mice were
5 challenged by intravenous injection with 2000 or 200 *P. berghei* sporozoites. This leads to infection of liver cells by the sporozoites. However, in the presence of a sufficiently strong T lymphocyte response against the intrahepatic parasite no viable parasite will leave the liver and no blood-stage parasites will be detectable. Blood films from challenged
10 mice were therefore assessed for parasites by microscopy 5-12 days following challenge.

BALB/c mice immunised twice with a mixture of two plasmid DNAs encoding the CS protein and the TRAP antigen, respectively, of *P. berghei* were not protected against sporozoite challenge. Mice immunised
15 twice with a mixture of recombinant MVA viruses encoding the same two antigens were not protected against sporozoite challenge. Mice immunised first with the two recombinant MVAs and secondly with the two recombinant plasmids were also not protected against sporozoite challenge. However, all 15 mice immunised first with the two plasmid
20 DNAs and secondly with the two recombinant MVA viruses were completely resistant to sporozoite challenge (Table 6 A and B).

To assess whether the observed protection was due to an immune response to the CS antigen or to TRAP or to both, groups of mice were then immunised with each antigen separately (Table 6 B). All 10
25 mice immunised first with the CS plasmid DNA and secondly with the CS MVA virus were completely protected against sporozoite challenge. Fourteen out of 16 mice immunised first with the TRAP plasmid DNA vaccine and secondly with the TRAP MVA virus were protected against sporozoite challenge. Therefore the CS antigen alone is fully protective

when the above immunisation regime is employed and the TRAP antigen is substantially protective with the same regime.

The good correlation between the induced level of CD8+ T lymphocyte response and the degree of protection observed strongly suggests that the CD8+ response is responsible for the observed protection. In previous adoptive transfer experiments it has been demonstrated that CD8+ T lymphocyte clones against the major CD8+ T cell epitope in the *P. berghei* CS protein can protect against sporozoite challenge. To determine whether the induced protection was indeed mediated by CD8+ T cells to this epitope we then employed a plasmid DNA and a recombinant MVA encoding only this nine amino acid sequence from *P. berghei* as a part of a string of epitopes (Table 6 B). (All the other epitopes were from micro-organisms other than *P. berghei*). Immunisation of 10 mice first with a plasmid encoding such an epitope string and secondly with a recombinant MVA also encoding an epitope string with the *P. berghei* CTL epitope led to complete protection from sporozoite challenge (Table 6 B). Hence the induced protective immune response must be the CTL response that targets this nonamer peptide sequence.

Table 6 Results of mouse challenge experiments using different combinations of DNA and MVA vaccine

	Immunisation 1	Immunisation 2	No. Infected/ No. challenged	%Protection
5	A. Antigen used: PbCSP + PbTRAP			
	DNA	DNA	5/5	0%
	MVA	MVA	9/10	10%
	DNA	MVA	0/5	100%
10	MVA	DNA	5/5	0%
	Control mice immunised with β -galactosidase			
	DNA	DNA	5/5	0%
	MVA	MVA	5/5	0%
15	DNA	MVA	5/5	0%
	MVA	DNA	5/5	0%
	B.			
	DNA (CSP +TRAP)	MVA (CSP +TRAP)	0/10	100%
20	DNA (CSP)	MVA (CSP)	0/10	100%
	DNA (TRAP)	MVA (TRAP)	2/16	88%
	DNA (epitope)	MVA (epitope)	0/11	100%
	DNA (beta-gal)	MVA (beta-gal)	6/7	14%
	none	none	9/10	10%

25

Table 6 Results of two challenge experiments (A and B) using different immunisation regimes of plasmid DNA and MVA as indicated. BALB/c mice were used in all cases. The immunisation doses were 50 μ g of plasmid DNA or 10^6 ffu of recombinant MVA. The interval between immunisations 1 and 2 was from 14-21 days in all cases. Challenges were performed at 18-29 days after the last immunisation by i.v. injection of 2000 *P. berghei* sporozoites and blood films assessed at 5, 8 and 10 days post challenge. CSP and TRAP indicate the entire *P. berghei* antigen and 'epitope' indicates the cassettes of epitopes shown in table 1 containing

30

only a single *P. berghei* K^d-restricted nonamer CTL epitope. Note that in experiment B immunisation with the epitope string alone yields 100% protection

Mice immunised twice with recombinant Ty-VLPs encoding pb9 were fully susceptible to infection. Similarly mice immunised twice with the recombinant MVA encoding the full CS protein were fully susceptible to infection. However, the mice immunised once with the Ty-VLP and subsequently once with the recombinant MVA showed an 85% reduction in malaria incidence when boosted with MVA expressing the full length CS protein, and 95% when MVA expressing the HM epitope string which includes pb9 was used to boost (Table 7).

Table 7 Results of challenge experiments using different immunisation regimes of Ty-VLPs and MVA

Immunisation 1	Immunisation 2	No. Infected/No.challenged	%Protection
Ty-CABDHFE	Ty- CABDHFE	7/8	13%
Ty-CABDH	MVA.PbCSP	2/13	85%
Ty- CABDHFE	MVA-NP	5/5	0%
MVA.PbCSP	MVA.PbCSP	6/6	0%
MVA.HM	Ty- CABDHFE	14/14	0%
Ty- CABDHFE	MVA.HM	1/21	95%
none	MVA.HM	8/8	0%
none	none	11/12	9%

Table 7 Results of challenge experiments using different immunisation regimes of Ty-VLPs and MVA as indicated. BALB/c mice were used in all cases. Immunisations were of 50 µg of Ty-VLP or 10⁷ ffu of recombinant MVA administered intravenously. The interval between immunisations 1 and 2 was from 14-21 days in all cases. Challenges were performed at 18-29 days after the last immunisation by i.v. injection of

2000 *P. berghei* sporozoites and blood films assessed at 5, 8 and 10 days post challenge. CSP indicates the entire *P. berghei* antigen. Ty-VLPs carried epitope cassettes CABDH or CABDHFE as described in table 1. MVA.HM includes cassettes CAB.

5 To determine whether the enhanced immunogenicity and protective efficacy observed by boosting with a recombinant MVA is unique to this particular vaccinia virus strain or is shared by other recombinant vaccinas the following experiment was performed. Mice were immunised with the DNA vaccine encoding *P. berghei* CS protein and boosted with
10 either (i) recombinant MVA encoding this antigen; (ii) recombinant wild-type vaccinia virus (Western Reserve strain) encoding the same antigen (Satchidanandam *et al.* 1991), or (iii) recombinant NYVAC (COPAK) virus (Lanar *et al.* 1996) encoding the same malaria antigen. The highest degree of protection was observed with boosting by the MVA recombinant,
15 80% (Table 8). A very low level of protection (10%) was observed by boosting with the wild-type recombinant vaccinia virus and a significant level of protection, 60%, by boosting with the NYVAC recombinant. Hence the prime-boost regime we describe induces protective efficacy with any non-replicating vaccinia virus strain. Both the MVA recombinant and
20 NYVAC were significantly ($P < 0.05$ for each) better than the WR strain recombinant.

Table 8 Challenge data results for DNA boosted with various vaccinia strain recombinants.

25

Immunisation 1	Immunisation 2	No. Infected/No. challenged	%Protection
DNA-beta gal.	MVA:NP	8/8	0%
DNA-CSP	MVA-CSP	2/10	80%
DNA-CSP	WR-CSP	9/10	10%
30 DNA-CSP	NYVAC-CSP	4/10	60%

Table 8 Results of a challenge experiment using different immunisation regimes of plasmid DNA and various vaccinia recombinants as indicated. BALB/c mice were used in all cases. The immunisation doses were 50 µg of plasmid DNA or 10⁶ ffu/pfu of recombinant MVA or 5 10⁴ ffu/pfu of recombinant wild type (WR) vaccinia or 10⁶ ffu/pfu of recombinant NYVAC. Because the WR strain will replicate in the host and the other strains will not, in this experiment a lower dose of WR was used. The interval between immunisations 1 and 2 was 23 days. Challenges were performed at 28 days after the last immunisation by i.v. injection of 10 2000 *P. berghei* sporozoites and blood films assessed at 7, 9 and 11 days post challenge. pbCSP indicates the entire *P. berghei* antigen and NP the nucleoprotein antigen of influenza virus (used as a control antigen). The first immunisation of group A mice was with the plasmid DNA vector expressing beta galactosidase but no malaria antigen.

15 In a further experiment shown in Table 8, mice were immunised with the DNA vaccine encoding *P. berghei* CS protein and boosted with either (i) recombinant MVA encoding this antigen; (ii) recombinant WR vaccinia virus encoding the same antigen or (iii) recombinant NYVAC (COPAK) virus encoding the same malaria antigen, 20 all at 10⁶ ffu/pfu. A high and statistically significant degree of protection was observed with boosting with recombinant NYVAC (80%) or recombinant MVA (66%). A low and non-significant level of protection (26%) was observed by boosting with the WR recombinant vaccinia virus (Table 9). MVA and NYVAC boosting each gave significantly more 25 protection than WR boosting ($P = 0.03$ and $P = 0.001$ respectively). These data re-emphasise that non-replicating pox virus strains are better boosting agents for inducing high levels of protection.

Table 9 Influence of different recombinant vaccinia strains on protection.

Immunisation 1 DNA	Immunisation 2	No. inf./ No. chall.	% protection
CSP	MVA.PbCSP	5/15	66
CSP	NYVAC.PbCSP	2/15	80
CSP	WR.PbCSP	11/15	26
β -galactosidase	MVA.NP	8/8	0

5

Table 9 Results of challenge experiments using different immunisation regimes of plasmid DNA and replication incompetent vaccinia recombinants as boosting immunisation. BALB/c mice were used in all cases. The immunisation doses were 50 μ g of plasmid DNA or 10⁶ ffu/pfu of recombinant MVA or recombinant wild type (WR) vaccinia or
 10 recombinant NYVAC. The interval between immunisations 1 and 2 was 23 days. Challenges were performed at 28 days after the last immunisation by i.v. injection of 2000 *P. berghei* sporozoites and blood films assessed at 7, 9 and 11 days post challenge. PbCSP indicates the entire *P. berghei*
 15 antigen and NP the nucleoprotein antigen of influenza virus (used as a control antigen). The control immunisation was with a plasmid DNA vector expressing β -galactosidase followed by MVA.NP.

Alternative routes for boosting immune responses with recombinant 20 MVA

Intravenous injection of recombinant MVA is not a preferred route for immunising humans and not feasible in mass immunisations.

Therefore different routes of MVA boosting were tested for their immunogenicity and protective efficacy.

Mice were primed with plasmid DNA i.m. Two weeks later they were boosted with MVA administered via the following routes: intravenous (i.v.), subcutaneous (s.c.), intraperitoneal (i.p.), intramuscular (i.p.) and intradermal (i.d.). Two weeks after this boost peptide-specific CD8+ T cells were determined in an ELISPOT assay. The most effective route which induced the highest levels were i.v. and i.d inoculation of MVA. The other routes gave moderate to poor responses (Figure 12).

Figure 12 shows frequencies of peptide-specific CD8+ T cells following different routes of MVA boosting. Results are shown as the number of spot-forming cells (SFC) per one million splenocytes. Mice were primed with plasmid DNA and two weeks later boosted with MVA via the indicated routes. The number of splenocytes specific for the SYIPSAEKI [SEQ ID NO: 67] peptide was determined in INF- γ ELISPOT assays two weeks after the last immunisation. Each bar represents the mean number of SFCs from three mice assayed individually.

Boosting via the i.v. route was compared with the i.d. and i.m route in a challenge experiment. The i.d route gave high levels of protection (80% protection). In the group of animals that were boosted via the i.m. route, 50% of the animals were protected. Complete protection was achieved with MVA boost administered i.v. (Table 10)

Table 10 Influence of the route of MVA administration on protective efficacy

Immunisation 1 DNA	Immunisation 2 MVA	No. infected/ No. challenged	% protection
CSP	CSP i.v.	*0/20	100
CSP	CSP i.d	2/10	80
CSP	CSP i.m.	5/10	50

Epitope	epitope i.v.	1/10	90
NP	NP i.v.	10/10	0

* culminative data from two independent experiments

Table 10 Results from challenge experiments using different routes of MVA boosting immunisation. Animals were primed by intramuscular plasmid DNA injection and two weeks later boosted with the indicated recombinant MVA (10^6 ffu/mouse) administered via the routes indicated. The mice were challenged 16 days after the last immunisation with 2000 P. berghei sporozoites and screened for blood stage parasitemia at day 8 and 10 post challenge. Epitope indicates the polypeptide string HM.

10

Alternative routes of DNA priming: The use of a gene gun to prime peptide specific CD8+ T cells

Gene gun delivery is described in detail in for example in Eisenbraun *et al.* DNA Cell Biol. 1993, 12: 791-797 and Degano *et al.* Vaccine 1998, 16: 394-398.

The mouse malaria challenge experiments described so far using plasmid DNA to prime an immune response used intramuscular injection of plasmid DNA. Intradermal delivery of plasmid DNA using a biolistic device is another route to prime specific CTL responses. Plasmid DNA is coated onto gold particles and delivered intradermally with a gene gun. Groups of mice (n=10) were immunised three times at two weeks intervals with the gene gun alone (4 µg/immunisation), immunised two times with the gene gun followed by an intravenous MVA.PbCSP boost or immunised intramuscularly with 50 µg of pTH.PbCSP and two weeks later boosted with MVA.PbCSP intravenously. Two weeks after the last immunisation the animals were challenged with 2000 sporozoites to assess protective efficacy of each immunisation regimen. In the group that received the intravenous MVA boost following two gene gun immunisations

one out of ten animals developed blood stage parasitemia (90% protection). Complete protection was observed with intramuscular DNA priming followed by MVA i.v boosting. Seven out of 10 animals that were immunised three times with the gene gun were infected. (30% protection)

5 (Table 11).

Immunisation 1	Immunisation 2	Immunisation 3	No. inf./ No. chall.	% protection
DNA				
gene gun DNA	gene gun DNA	gene gun DNA	7/10	30
gene gun DNA	gene gun DNA	MVA.PbCSP	1/10	90
-	DNA i.m	MVA.PbCSP	0/10	100
Naïve			10/10	0

Table 11 Results of challenge experiments comparing different routes of DNA priming (intradermally by gene gun versus intramuscular needle

10 of DNA priming (intradermally by gene gun versus intramuscular needle injection). Groups of BALB/c mice (n=10) were immunised as indicated. Each gene gun immunisation delivered 4 µg of plasmid DNA intraepidermally. For i.m. immunisations 50 µg of plasmid DNA were injected. Twenty days after the last immunisation mice were challenged as

15 described previously.

Highly susceptible C57BL/6 mice are protected

C57BL/6 mice are very susceptible to *P. berghei* sporozoite challenge. C57BL/6 mice were immunised using the DNA-MVA prime boost regime

20 with both pre-erythrocytic antigens PbCSP and PbTRAP, and challenged with either 200 or 1000 infectious sporozoites per mouse. (Two hundred sporozoites corresponds to more than twice the dose required to induce infection in this strain). All ten mice challenged with 200 sporozoites showed sterile immunity. Even the group challenged with 1000

sporozoites, 60% of the mice were protected (Table 12). All the naïve C57BL/6 mice were infected after challenge.

Table 12 Protection of C57BL/6 mice from sporozoite challenge

	No. animals inf./ No. challenged	% protection
1000 sporozoites		
DNA followed by MVA	4/10	60
Naïve	5/5	0
200 sporozoites		
DNA followed by MVA	0/10	100
Naïve	5/5	0

Table 12 Results of a challenge experiment using C57BL/6 mice. Animals were immunised with PbCSP and PbTRAP using the DNA followed by MVA prime boost regime. Fourteen days later the mice were challenged with *P. berghei* sporozoites as indicated.

EXAMPLE 4

Protective efficacy of the DNA-priming/MVA-boosting regimen in two further disease models in mice

Following immunogenicity studies, the protective efficacy of the DNA-priming MVA-boosting regimen was tested in two additional murine challenge models. The two challenge models were the P815 tumour model and the influenza A virus challenge model. In both model systems CTL have been shown to mediate protection.

P815 tumour challenges:

Groups (n = 10) of DBA/2 mice were immunised with a combination of DNA followed by MVA expressing a tumour epitope string or the HM epitope

string. Two weeks after the last immunisation the mice were challenged intravenously with 10^5 P815 cells. Following this challenge the mice were monitored regularly for the development of tumour-related signs and survival.

5 Figure 13 shows the survival rate of the two groups of mice. Sixty days after challenge eight out of ten mice were alive in the group immunised with the tumour epitopes string. In the group immunised with the HM epitope string only 2 animals survived. This result is statistically significant: 2/10 vs 8/10 chi-squared = 7.2. $P = 0.007$. The onset of death
10 in the groups of animals immunised with the tumour epitope string is delayed compared to the groups immunised with the HM epitope string.

Influenza virus challenges:

Groups of BALB/c mice were immunised with three gene gun
15 immunisations with plasmid DNA, two intramuscular plasmid DNA injections, one i.m. DNA injection followed by one MVA.NP boost i.v. or two gene gun immunisations followed by one MVA.NP boost i.v. Plasmid DNA and recombinant MVA expressed the influenza virus nucleoprotein. Two weeks after the last immunisation the mice were challenged intranasally
20 with 100 HA of influenza A/PR/8/34 virus. The animals were monitored for survival daily after challenge.

Complete protection was observed in the following groups of animals

- two DNA gene gun immunisations followed by one MVA.NP boost i.v.,
- one i.m. DNA injection followed by one MVA.NP boost i.v.
- 25 • two i.m. DNA injections.

In the group of animals immunised three times with the gene gun 71% of the animals survived (5/7) and this difference from the control group was not significant statistically ($P > 0.05$). In the naive group 25% of
30 the animals survived (Figure 14) and this group differed significantly ($P < 0.05$) for the two completely protected groups.

Figure 14 shows results of an influenza virus challenge experiment. BALB/c mice were immunised as indicated. GG = gene gun immunisations, im = intramuscular injection, iv = intravenous injection. Survival of the animals was monitored daily after challenge.

5 In a second experiment groups of 10 BALB/c mice were immunised with MVA.NP i.v. alone, three times with the gene gun, two times with the gene gun followed by one MVA.NP boost i.v. and two i.m injections of V1J-NP followed by one MVA.NP boost. Two weeks after the last immunisation the mice were challenged with 100 HA units of influenza
10 A/PR/8/34 virus.

Complete and statistically significant protection was observed in the following groups of animals:

- two gene gun immunisations followed by one MVA.NP boost,
- two i.m injections of V1J-NP followed by one MVA.NP boost.

15 In the group receiving one MVA.NP i.v., 30% (3 out of 10) of animals survived. In the group immunised with a DNA vaccine delivered by the gene gun three times, 70% of the animals were protected but this protection was not significantly different from the naïve controls. In this challenge experiment 40% (4 out of 10) of the naive animals survived the
20 challenge.

EXAMPLE 5

Immunogenicity studies in non-human primates

25 **Immunogenicity and protective efficacy of the prime boost regimen in non-human primates.**

In order to show that the strong immunogenicity of the DNA priming/MVA boosting regime observed in mice translates into strong immunogenicity in primates, the regimen was tested in macaques. The
30 vaccine consisted of a string of CTL epitopes derived from HIV and SIV sequences (Figure 2), in plasmid DNA or MVA, denoted DNA.H and

MVA.H respectively. The use of defined CTL epitopes in a polyepitope string allows testing for SIV specific CTL in macaques. Due to the MHC class I restriction of the antigenic peptides, macaques were screened for their MHC class I haplotype and Mamu-A*01-positive animals were
5 selected for the experiments described.

Three animals (CYD, DI and DORIS) were immunised following this immunisation regimen:

	week 0	DNA (8µg, i.d., gene gun)
10	week 8	DNA (8µg, i.d., gene gun)
	week 17	MVA (5 x 10 ⁸ pfu, i.d.)
	week 22	MVA (5 x 10 ⁸ pfu, i.d.)

Blood from each animal was drawn at weeks 0, 2, 5, 8, 10,
15 11, 17, 18, 19, 21, 22, 23, 24 and 25 of the experiment. The animals were monitored for induction of CTL using two different methods. PBMC isolated from each bleed were re-stimulated *in vitro* with a peptide encoded in the epitope string and tested for their ability to recognise autologous peptide-loaded target cells in a chromium release cytotoxicity assay. Additionally,
20 freshly isolated PBMC were stained for antigen specific CD8+ T cells using tetramers.

Following two gene gun immunisations very low levels of CTL were detected using tetramer staining (Figure 15). Two weeks after the first MVA boosting, all three animals developed peptide specific CTL as
25 detected by tetramer staining (Figure 15). This was reflected by the detection of moderate CTL responses following *in vitro* restimulation (Figure 16, week 19). The second boost with MVA.H induced very high levels of CD8+, antigen specific T cells (Figure 15) and also very high levels of peptide specific cytotoxic T cells (Figure 16, week 23).

30 Figure 15 shows detection of SIV-specific MHC class I-restricted CD8+ T cells using tetramers. Three Mamu-A*A01-positive

macaques were immunised with plasmid DNA (gene gun) followed by MVA boosting as indicated. Frequencies of Mamu-A*01/CD8 double-positive T cells were identified following FACS analysis. Each bar represents the percentage of CD8+ T cells specific for the Mamu-A*01/gag epitope at the indicated time point. One percent of CD8 T cells corresponds to about 5000/10⁶ peripheral blood lymphocytes. Thus the levels of epitope-specific CD8 T cells in the peripheral blood of these macaques are at least as high as the levels observed in the spleens of immunised and protected mice in the malaria studies.

Figure 16 shows CTL induction in macaques following DNA/MVA immunisation. PBMC from three different macaques (CYD, DI and DORIS) were isolated at week 18, 19 and 23 and were restimulated with peptide CTPYDINQM [SEQ ID NO: 54] *in vitro*. After two restimulations with peptide CTPYDINQM [SEQ ID NO: 54] the cultures were tested for their lytic activity on peptide-pulsed autologous target cells. Strong CTL activity was observed.

EXAMPLE 6

Immunogenicity and Challenge Studies in Chimpanzees

To show that a similar regime of initial immunisation with plasmid DNA and subsequent immunisation with recombinant MVA can be effective against *Plasmodium falciparum* malaria in higher primates an immunisation and challenge study was performed with two chimpanzees. Chimp H1 received an initial immunisation with 500 µg of a plasmid expressing *Plasmodium falciparum* TRAP from the CMV promoter without intron A, CMV-TRAP. Chimp H2 received the same dose of CMV-LSA-1, which expresses the C-terminal portion of the LSA-1 gene of *P. falciparum*. Both chimps received three more immunisations over the next 2 months, but with three plasmids at each immunisation. H1 received CMV-TRAP as before, plus pTH-TRAP, which expresses TRAP using the CMV promoter

with intron A, leading to a higher expression level. H1 also received RSV-LSA-1, which expresses the C-terminal portion of LSA-1 from the RSV promoter. H2 received CMV-LSA-1, pTH-LSA-1 and RSV-TRAP at the second, third and fourth immunisations. The dose was always 500 µg of
5 each plasmid.

It was subsequently discovered that the RSV plasmids did not express the antigens contained within them, so H1 was only immunised with plasmids expressing TRAP, and H2 with plasmids expressing LSA-1.

10 Between and following these DNA immunisations assays of cellular immune responses were performed at several time points, the last assay being performed at three months following the fourth DNA immunisation, but no malaria-specific T cells were detectable in either ELISPOT assays or CTL assays for CD8+ T cells.

15 Both animals were subsequently immunised with three doses of 10^8 ffu of a recombinant MVA virus encoding the *P. falciparum* TRAP antigen over a 6 week period. Just before and also following the third recombinant MVA immunisation T cell responses to the TRAP antigen were detectable in both chimpanzees using an ELISPOT assay to whole
20 TRAP protein bound to latex beads. This assay detects both CD4+ and CD8+ T cell responses. Specific CD8+ T responses were searched for with a series of short 8-11 amino acid peptides in both immunised chimpanzees. Such analysis for CD8+ T cell responses indicated that CD8+ T cells were detectable only in the chimpanzee H1. The target
25 epitope of these CD8+ T lymphocytes was an 11 amino acid peptide from TRAP, tr57, of sequence KTASCGVWDEW [SEQ ID NO: 78]. These CD8+ T cells from H1 had lytic activity against autologous target cells pulsed with the tr57 peptide and against autologous target cells infected with the recombinant PfTRAP-MVA virus. A high precursor frequency of
30 these specific CD8+ T cells of about 1 per 500 lymphocytes was detected

in the peripheral blood of this chimpanzee H1 using an ELISPOT assay two months following the final MVA immunisation. No specific CD8+ T cell response was clearly detected in the chimpanzee H2, which was not primed with a plasmid DNA expressing TRAP.

5 Two months after the third PfTRAP-MVA immunisation challenge of H1 and H2 was performed with 20,000 sporozoites, a number that has previously been found to yield reliably detectable blood stage infection in chimpanzees 7 days after challenge (Thomas *et al.* 1994 and unpublished data). The challenge was performed with the NF54 strain of
10 *Plasmodium falciparum*. This is of importance because the TRAP sequence in the plasmid DNA and in the recombinant MVA is from the T9/96 strain of *P. falciparum* which has numerous amino acid differences to the NF54 TRAP allele (Robson *et al.* 1990). Thus, this sporozoite challenge was performed with a heterologous rather than homologous
15 strain of parasite. In the chimpanzee H2 parasites were detectable in peripheral blood as expected 7 days after sporozoite challenge using *in vitro* parasite culture detection. However, in H1 the appearance of blood stage parasites in culture from the day 7 blood samples was delayed by three days consistent with some immune protective effect against the liver-
20 stage infection. In studies of previous candidate malaria vaccines in humans a delay in the appearance of parasites in the peripheral blood has been estimated to correspond to a substantial reduction in parasite density in the liver (Davis *et al.* 1989). Thus the chimpanzee H1, immunised first with *P. falciparum* TRAP plasmid DNA and subsequently with the same
25 antigen expressed by a recombinant MVA virus showed a strong CD8+ T lymphocyte response and evidence of some protection from heterologous sporozoite challenge.

DISCUSSION

These examples demonstrate a novel regime for immunisation against malaria which induces high levels of protective CD8+ T cells in rodent models of human malaria infection. Also demonstrated is an unprecedented complete protection against sporozoite challenge using subunit vaccines (36 out of 36 mice protected in Table 6 using DNA priming and MVA boosting with the CS epitope containing vaccines). Induction of protective immune responses using the DNA priming/MVA boosting regimen was demonstrated in two additional mouse models of viral infection influenza A model and cancer (P815 tumour model). More importantly for vaccines for use in humans this immunisation regimen is also highly immunogenic for CD8+ T cells in primates. Strong SIV-gag-specific CTL were induced in 3 out of 3 macaques with plasmid DNA and MVA expressing epitope strings. The levels induced are comparable to those found in SIV-infected animals. The data from the chimpanzee studies indicate that the same immunisation regime can induce a strong CD8+ T lymphocyte response against *P. falciparum* in higher primates with some evidence of protection against *P. falciparum* sporozoite challenge.

Ty-VLPs have previously been reported to induce good levels of CD8+ T cell responses against the *P. berghei* rodent malaria (Allsopp *et al.* 1995) but alone this construct is not protective. It has now been found that subsequent immunisation with recombinant MVA boosts the CD8+ T cell response very substantially and generates a high level of protection (Table 7).

Recombinant MVA viruses have not been assessed for efficacy as malaria vaccines previously. Recombinant MVA alone was not significantly protective, nor was priming with recombinant MVA followed by a second immunisation with recombinant plasmid DNA. However, a second immunisation with the recombinant MVA following an initial immunisation with either Ty-VLPs or plasmid DNA yielded impressive

levels of protection. Non-recombinant MVA virus has been safely used to vaccinate thousands of human against smallpox and appears to have an excellent safety profile. The molecular basis of the increased safety and immunogenicity of this strain of vaccinia virus is being elucidated by
5 detailed molecular studies (Meyer *et al.* 1991; Sutter *et al.* 1994).

Plasmid DNA has previously been tested as a malaria vaccine for the *P. yoelii* rodent malaria. High levels of, but not complete, protection is seen in some strains but in other strains of mice little or no protection was observed even after multiple immunisations (Doolan *et al.*
10 1996). Although plasmid DNA has been proposed as a method of immunisation against *P. falciparum*, success has not previously been achieved. The evidence provided here is the first evidence to show that plasmid DNA may be used in an immunisation regime to induce protective immunity against the human malaria parasite *P. falciparum*.

15 A similar regime of immunisation to the regime demonstrated herein can be expected to induce useful protective immunity against *P. falciparum* in humans. It should be noted that five of the vaccine constructs employed in these studies to induce protective immunity in rodents or chimpanzees contain *P. falciparum* sequences and could
20 therefore be used for human immunisation against *P. falciparum*. These are: 1. The *P. falciparum* TRAP plasmid DNA vaccine. 2. The *P. falciparum* TRAP recombinant MVA virus. 3. The Ty-VLP encoding an epitope string of numerous *P. falciparum* epitopes, as well as the single *P. berghei* CTL epitope. 4. The plasmid DNA encoding the same epitope
25 string as 3. 5. The recombinant MVA encoding the longer HM epitope string including many of the malaria epitopes in 3 and 4. Similarly the plasmid DNAs and MVA encoding HIV epitopes for human class I molecules could be used in either prophylactic or therapeutic immunisation against HIV infection.

These studies have provided clear evidence that a novel sequential immunisation regime employing a non-replicating or replication-impaired pox virus as a boost is capable of inducing a strong protective CD8+ T cell response against the malaria parasite. The examples demonstrate clearly a surprising and substantial enhancement of CD8+ T cell responses and protection compared to replicating strains of pox viruses. Because there is no reason to believe that the immunogenicity of CD8+ T cell epitopes from the malaria parasite should differ substantially from CD8+ T cell epitopes in other antigens it is expected that the immunisation regime described herein will prove effective at generating CD8+ T cell responses of value against other diseases. The critical step in this immunisation regimen is the use of non-replicating or replication-impaired recombinant poxviruses to boost a pre-existing CTL response. We have shown that CTL responses can be primed using different antigen delivery systems such as a DNA vaccine i.d. and i.m, a recombinant Ty-VLP, a recombinant adenovirus and irradiated sporozoites. This is supported by the data presented on the generation of a CD8+ T cell response against HIV, influenza virus and tumours. Amongst several known examples of other diseases against which a CD8+ T cell immune response is important are the following: infection and disease caused by the viruses HIV, herpes simplex, herpes zoster, hepatitis C, hepatitis B, influenza, Epstein-Barr virus, measles, dengue and HTLV-1; by the bacteria *Mycobacterium tuberculosis* and *Listeria* sp.; and by the protozoan parasites *Toxoplasma* and *Trypanosoma*. Induction of protective CTL responses against influenza A virus has been demonstrated in Figure 14. Furthermore, the immunisation regime described herein is expected to be of value in immunising against forms of cancer where CD8+ T cell responses plays a protective role. The induction of protective CTL responses using the DNA prime MVA boost regime against tumours is

shown in Figure 13. Specific examples in humans include melanoma, cancer of the breast and cancer of the colon.

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>12</u> , line <u>10 - 13</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution European Collection of Animal Cell Cultures (CAMR)	
Address of depositary institution (including postal code and country) Salisbury Wiltshire SP4 0JG United Kingdom	
Date of deposit 5 June 1997	Accession Number V97060511
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>	
In respect of all designated States to which such action is possible and to the extent that it is legally permissible under the law of the designated State, it is requested that a sample of the deposited microorganism be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), UK Patent Rules 1995, Schedule 2, Paragraph 3, Australian Regulation 3.25(3), Danish	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

C. ADDITIONAL INDICATIONS (continued)

Patents Act Sections 22 and 33(3), Icelandic Patents Act Sections 22 and 33(3), Norwegian Patents Act Sections 22 and 33(3) and generally similar provisions *mutatis mutandis* for any other designated State.

CLAIMS

1. A kit for generating a protective CD8+ T cell immune
5 response against at least one target antigen, which kit comprises:
 - (i) a priming composition comprising a source of one or more CD8+ T cell epitopes of the target antigen, together with a pharmaceutically acceptable carrier; and
 - (ii) a boosting composition comprising a source of one or more CD8+ T
10 cell epitopes of the target antigen, including at least one CD8+ T cell epitope which is the same as a CD8+ T cell epitope of the priming composition, wherein the source of CD8+ T cell epitopes is a non-replicating or replication-impaired recombinant poxvirus vector, together with a pharmaceutically acceptable carrier;
- 15 with the proviso that if the source of epitopes in (i) is a viral vector, the viral vector in (ii) is derived from a different virus.
2. The kit according to claim 1, wherein the source of CD8+ T cell epitopes in (i) is a non-viral vector or a non-replicating or replication-impaired viral vector.
- 20 3. The kit according to claim 1 or claim 2, wherein the source of CD8+ T cell epitopes in (i) is not a poxvirus vector.
4. The kit according to claim 2 or claim 3, wherein the source of CD8+ T cell epitopes in (i) is DNA or RNA.
5. The kit according to claim 4, wherein the source of epitopes
25 in (i) is a recombinant DNA plasmid.
6. The kit according to claim 4 or claim 5, further comprising GM-CSF as an adjuvant for (i).
7. The kit according to any one of claims 1 to 6, wherein the
30 source of CD8+ T cell epitopes in (i) encodes or comprises the target antigen.

8. The kit according to any one of claims 4 to 6, wherein the source of epitopes in (i) encodes a single CD8+ T cell epitope or a recombinant string of two or more CD8+ T cell epitopes.
9. The kit according to any one of claims 1 to 3, wherein the
5 source of epitopes in (i) is a peptide, polypeptide, protein, polyprotein or particle comprising two or more CD8+ T cell epitopes, present in a recombinant string of CD8+ T cell epitopes or in a target antigen.
10. The kit according to claim 9, wherein the source of CD8+ T cell epitopes in (i) is a recombinant protein particle such as a Ty virus-like
10 particle (VLP).
11. The kit according to any one of claims 1 to 3, wherein the source of epitopes in (i) is a recombinant adenovirus vector.
12. The kit according to any one of claims 1 to 11, wherein the source of CD8+ T cell epitopes in (ii) is a recombinant vaccinia virus vector.
- 15 13. The kit according to claim 12, wherein the recombinant vaccinia virus vector is of the vaccinia virus strain Modified Virus Ankara (MVA), or a strain derived therefrom.
14. The kit according to claim 12, wherein the recombinant vaccinia virus vector is of the strain NYVAC or a strain derived therefrom.
- 20 15. The kit according to any one of claims 1 to 11, wherein the source of CD8+ T cell epitopes in (ii) is a recombinant avipox vector such as canary pox or fowl pox or strains derived therefrom such as ALVAC.
16. The kit according to any one of claims 1 to 15, for generating a protective immune response against a pathogen or tumour comprising
25 the target antigen.
17. The kit according to claim 16, for generating a protective immune response against a malaria pathogen such as *Plasmodium falciparum*.

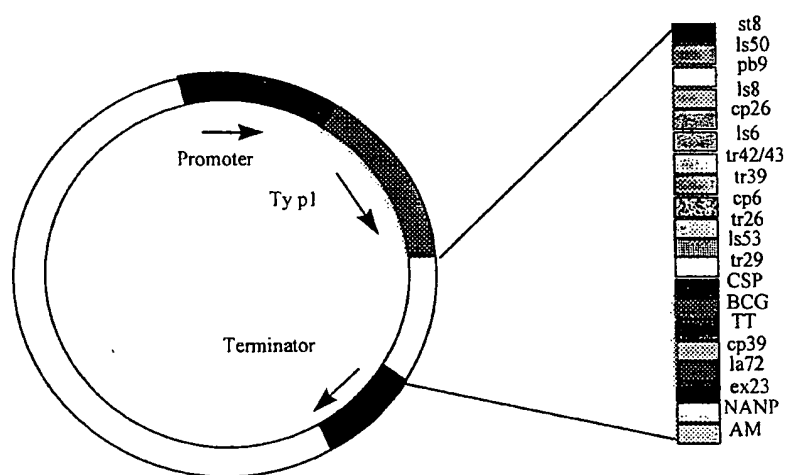
18. A kit according to claim 17, wherein the CD8+ T cell epitopes in or encoded by (i) include one or more malaria epitopes from the list given in table 1.
19. The kit according to claim 18, wherein the CD8+ T cell
5 epitopes in (i) include all of the epitopes given in table 1.
20. The kit according to claim 16, for generating an immune response against HIV.
21. The kit according to claim 20, wherein the CD8+ T cell epitopes in or encoded by (i) include one or more HIV epitopes from the list
10 given in table 2.
22. The kit according to claim 20, wherein the CD8+ T cell epitopes in or encoded by (i) include all of the epitopes given in table 2.
23. The kit according to any one of claims 1 to 22, wherein the priming and boosting compositions are identical in that both contain the
15 source of epitopes in (i) and the source of epitopes in (ii).
24. The kit according to any one of claims 1 to 23, wherein the priming composition and/or the boosting composition is in particulate form suitable for delivery by means of a gene gun.
25. A method for generating a protective CD8+ T cell immune
20 response against at least one target antigen, which method comprises administering at least one dose of component (i), followed by at least one dose of component (ii) of the kit according to any one of claims 1 to 24.
26. A method for generating a protective CD8+ T cell immune response against a pathogen or tumour, which method comprises
25 administering at least one dose of a recombinant DNA plasmid encoding at least one CD8+ T cell epitope or antigen of the pathogen or cancer, followed by at least one dose of a recombinant non-replicating or replication-impaired pox virus encoding the same epitope or antigen.
27. The method according to claim 25, wherein the recombinant
30 vaccinia virus is a recombinant MVA vector.

28. A method for generating a protective CD8+ T cell immune response against a pathogen or tumour, which method comprises administering at least one dose of a recombinant protein or particle comprising at least one epitope or antigen of the pathogen or cancer,
5 followed by at least one dose of a recombinant MVA vector encoding the same epitope or antigen.
29. The method according to any one of claims 26 to 28, for generating a protective CD8+ T cell immune response against malaria such as *P. falciparum* malaria.
- 10 30. The method according to any one of claims 26 to 28, for generating a protective CD8+ T cell immune response against HIV.
31. The method according to any one of claims 25 to 30, wherein (ii) is delivered intravenously, intraepidermally or intradermally.
32. A medicament for boosting a primed CD8+ T cell response
15 against at least one target antigen, comprising a source of one or more CD8+ T cell epitopes of the target antigen, wherein the source of CD8+ T cell epitopes is a non-replicating or a replication-impaired recombinant poxvirus vector, and a pharmaceutically acceptable carrier.
33. The medicament according to claim 32, wherein the vector is
20 a vaccinia virus vector such as MVA.
34. The medicament according to claim 32 or 33, for boosting a naturally primed CD8+ T cell response against malaria.
35. A method of boosting a primed CD8+ T cell immune response, which method comprises administering a medicament according
25 to any one of claims 32 to 34.
36. The use of a recombinant non-replicating or replication-impaired pox virus vector in the manufacture of a medicament for boosting a CD8+ T cell immune response.
37. The use of an MVA vector in the manufacture of a
30 medicament for boosting a CD8+ T cell immune response.

38. An epitope string comprising the amino acid sequences listed in table 1.
39. A recombinant Ty-VLP comprising the epitope string according to claim 38, for immunising against malaria.
- 5 40. A recombinant DNA plasmid or recombinant non-replicating or replication-impaired pox virus encoding the epitope string according to claim 38, for immunising against malaria.
41. A recombinant DNA plasmid or recombinant non-replicating or replication-impaired pox virus encoding the *P. falciparum* antigen TRAP,
10 for immunising against malaria.
42. A recombinant vaccinia virus according to claim 40 or claim 41, of the MVA strain.
43. An epitope string comprising the amino acid sequences listed in table 2.
- 15 44. A recombinant polypeptide comprising a whole or substantially whole protein antigen such as TRAP and a string of two or more epitopes such as CTL epitopes from malaria.

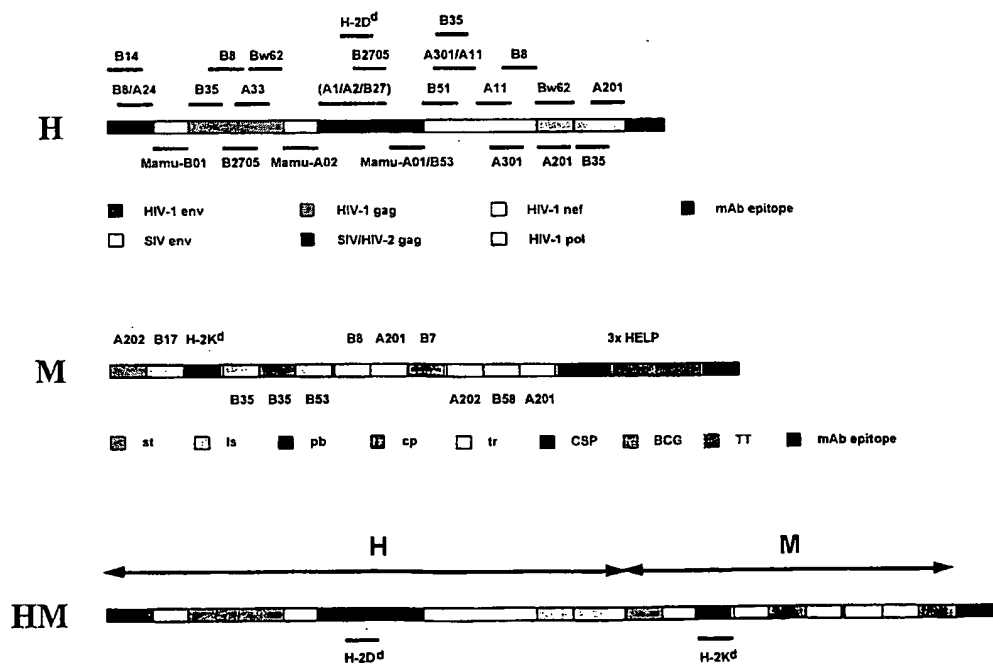
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Figure 1



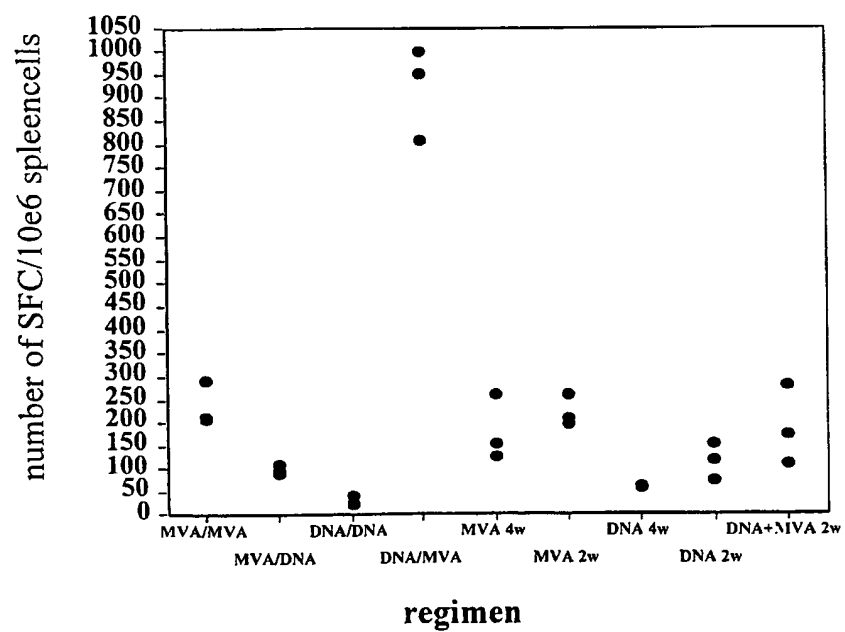
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Figure 2



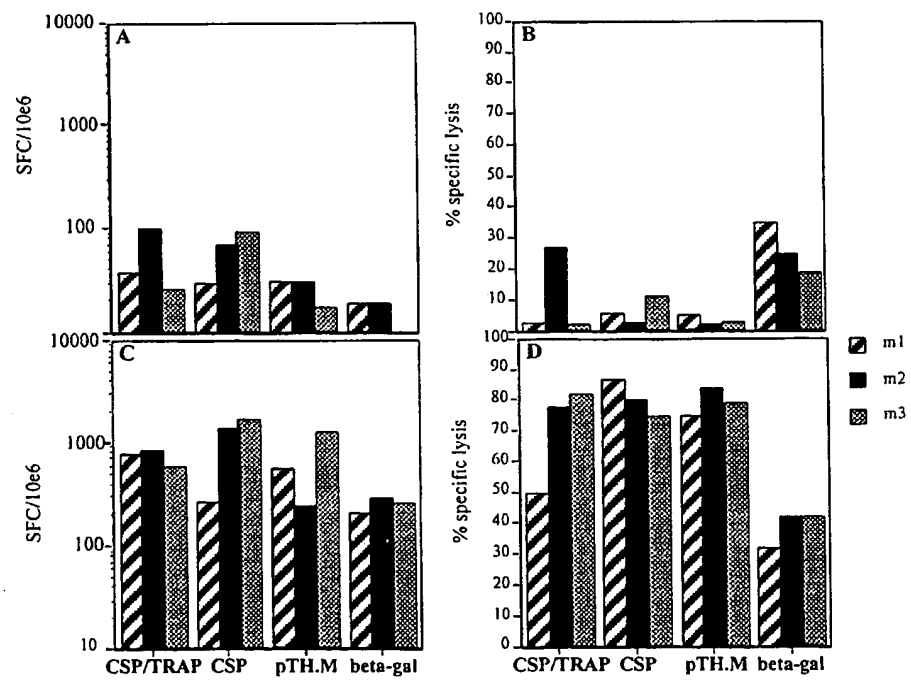
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Figure 3



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Figure 4



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Figure 5

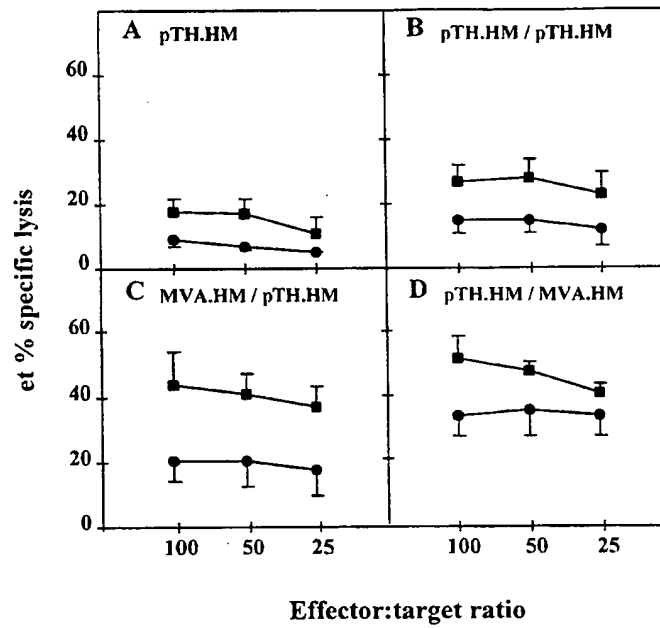
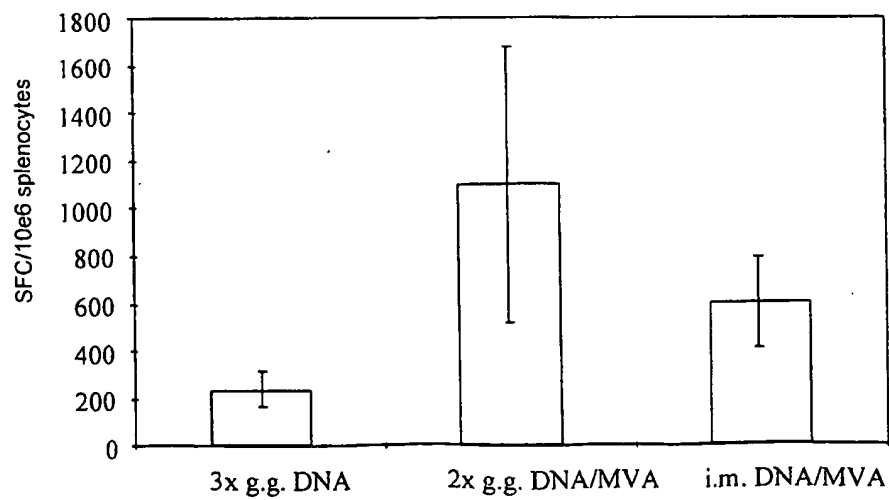
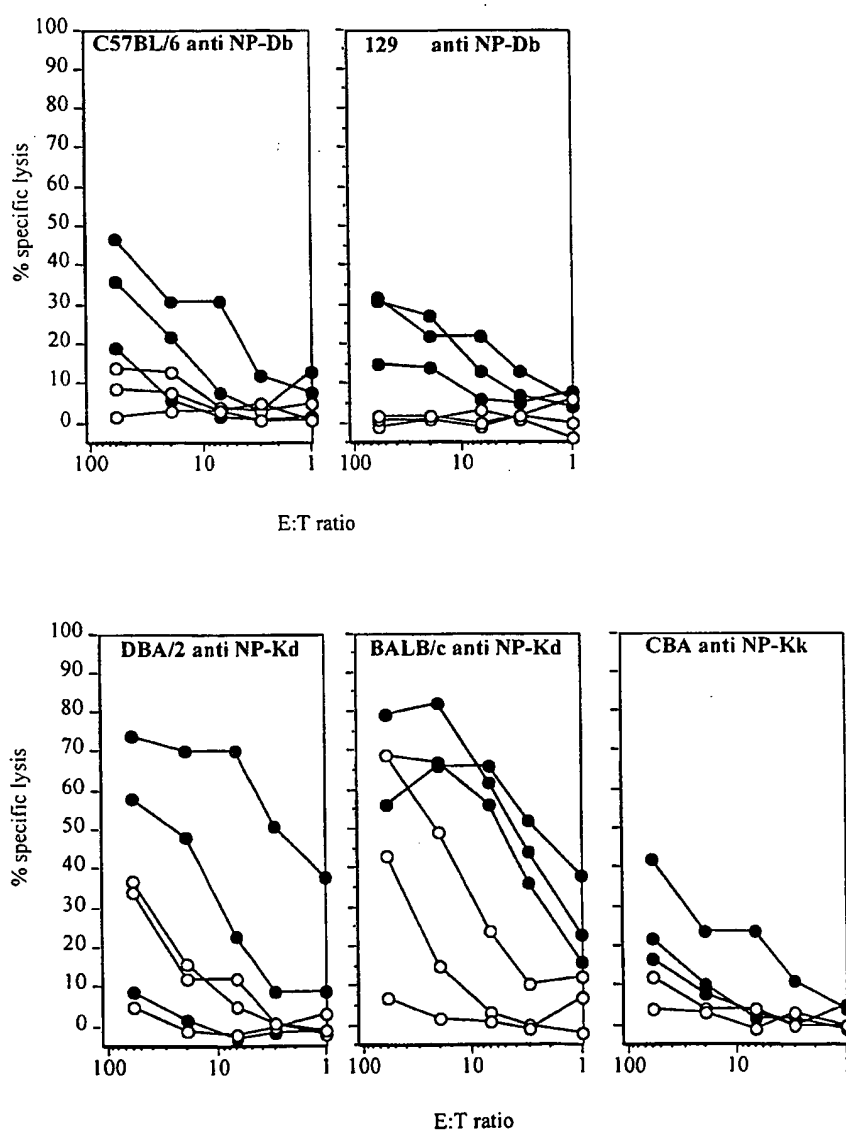


Figure 6



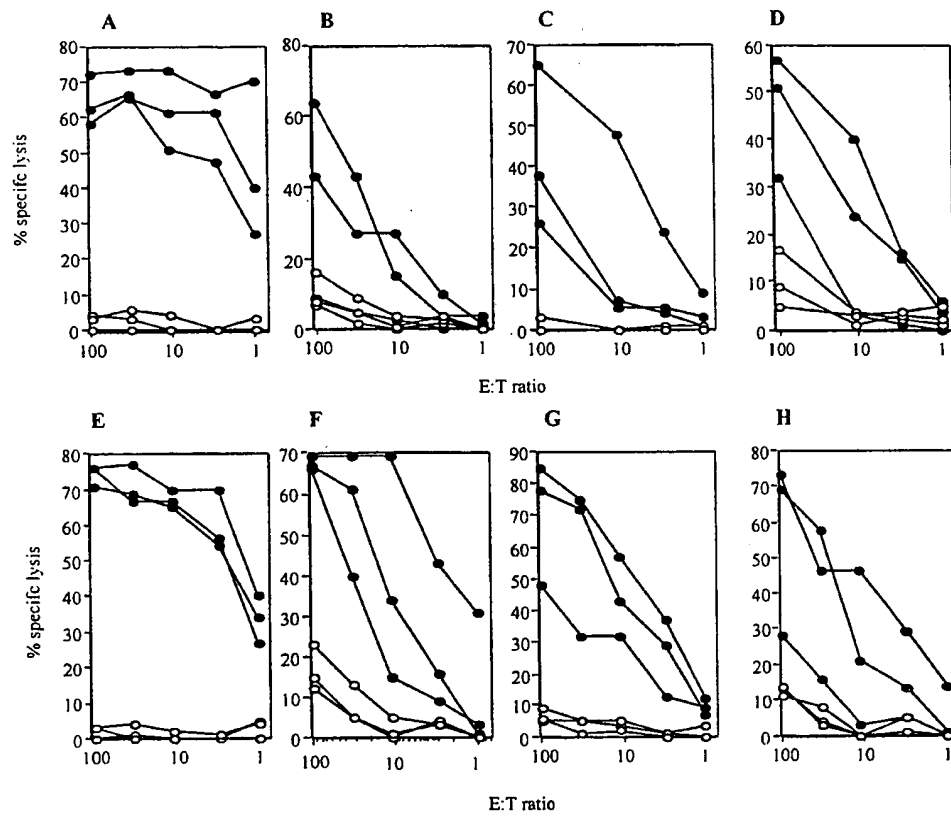
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Figure 7



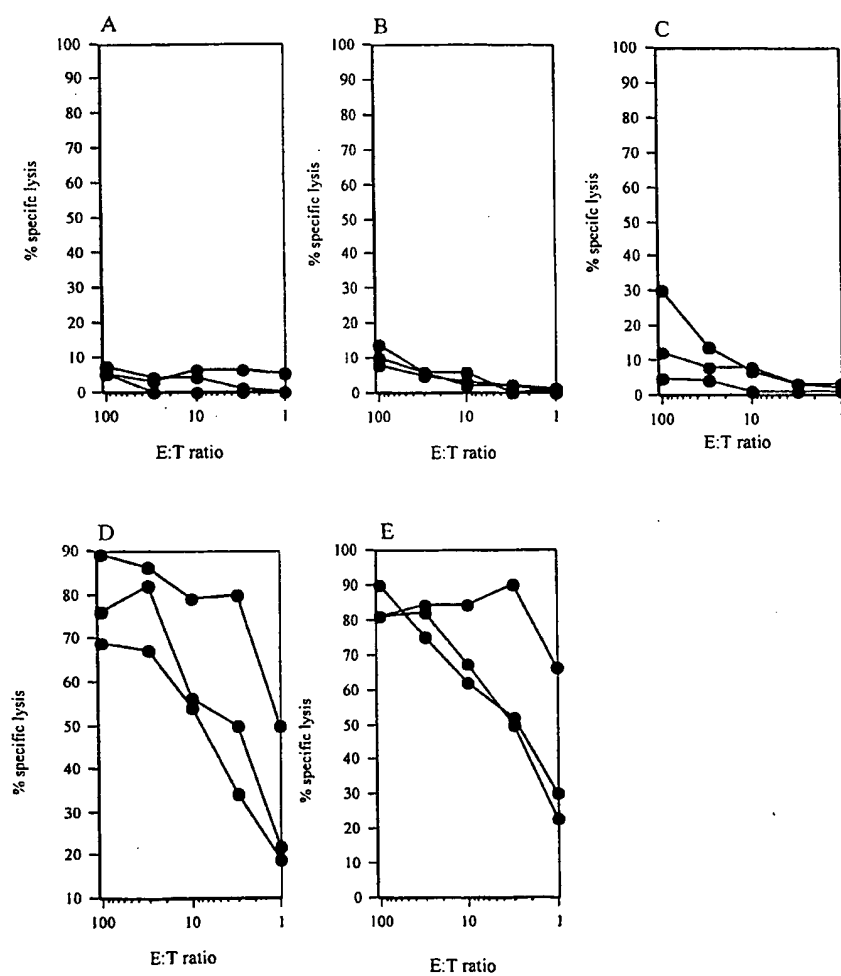
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Figure 8



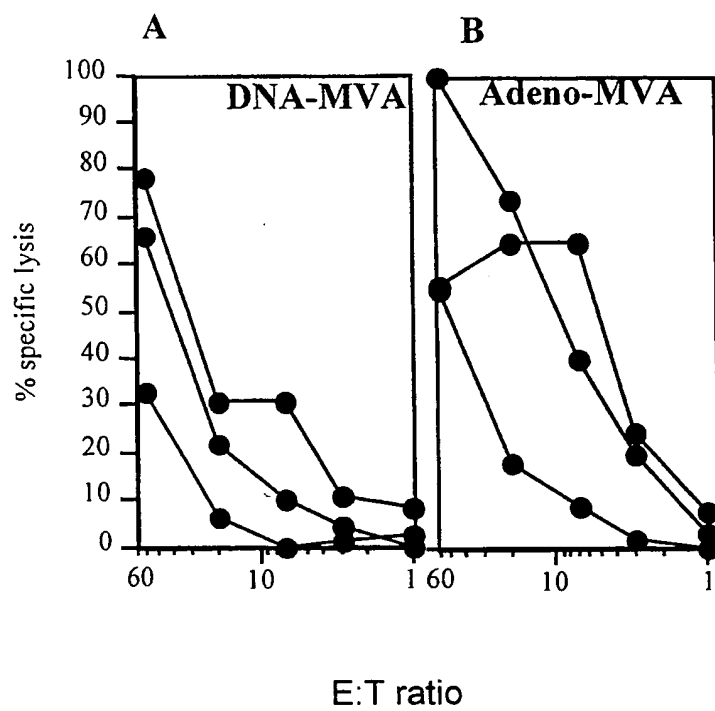
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Figure 9



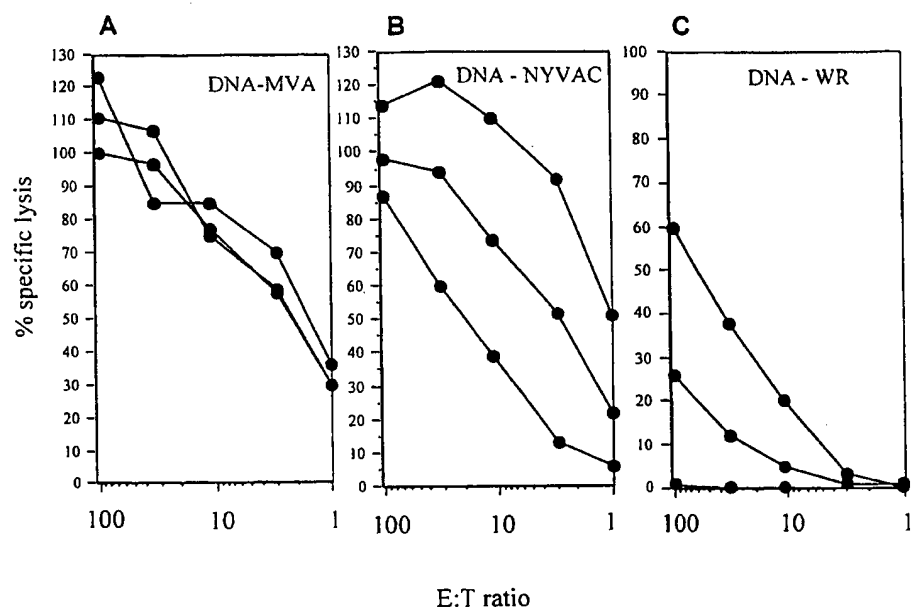
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Figure 10



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Figure 11



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Figure 12

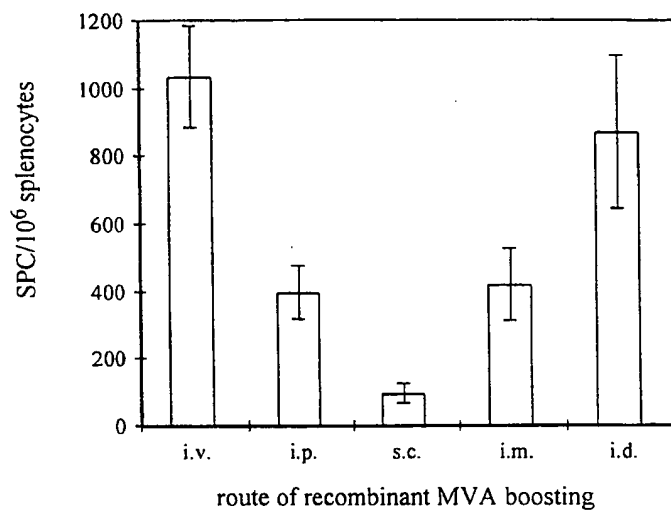
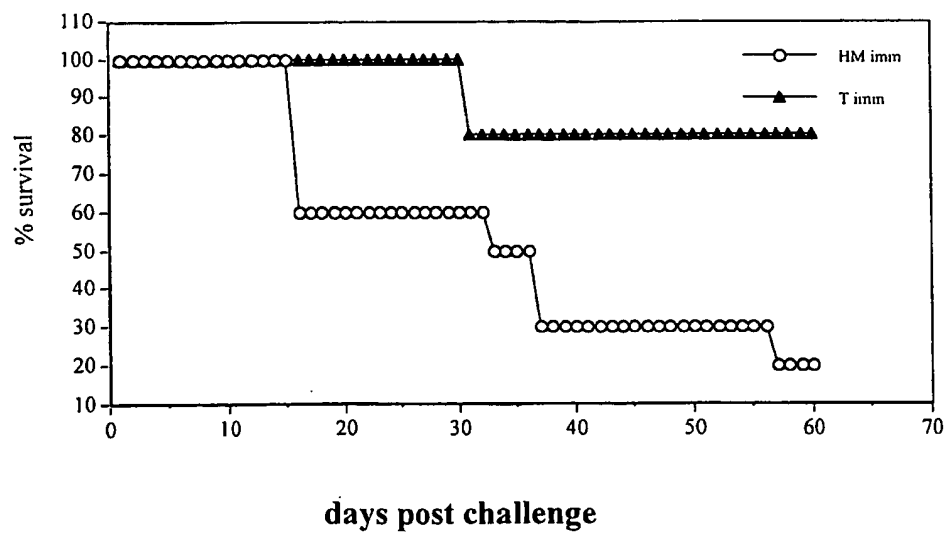
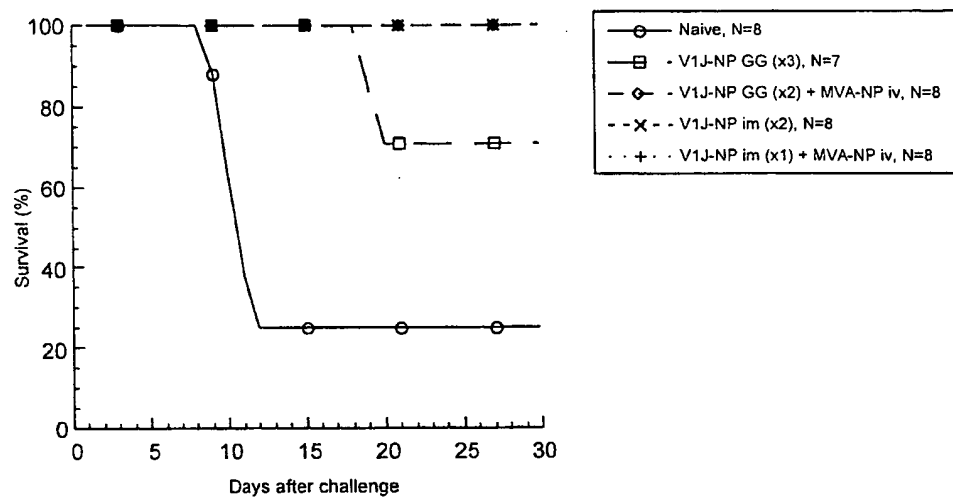


Figure 13



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Figure 14



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Figure 15

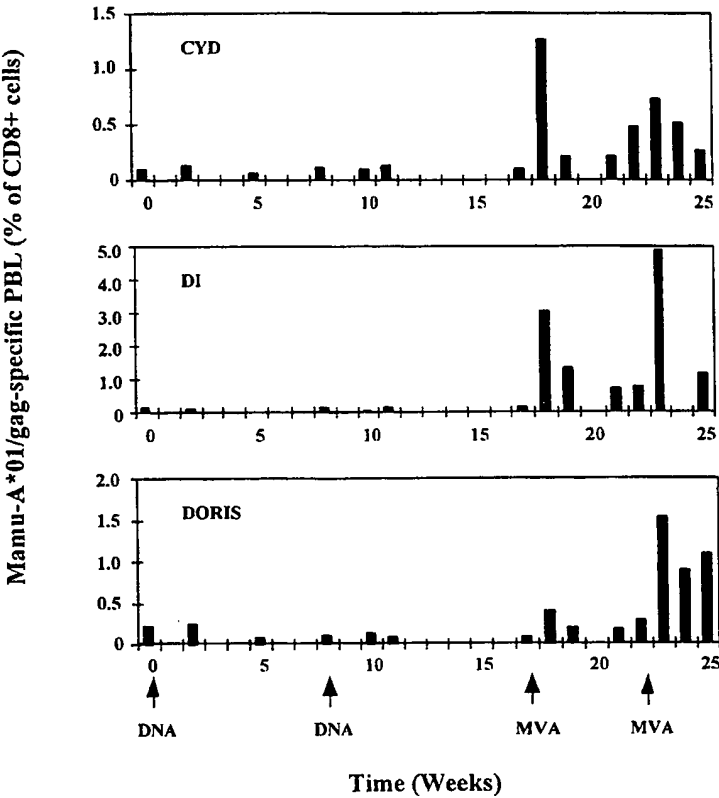
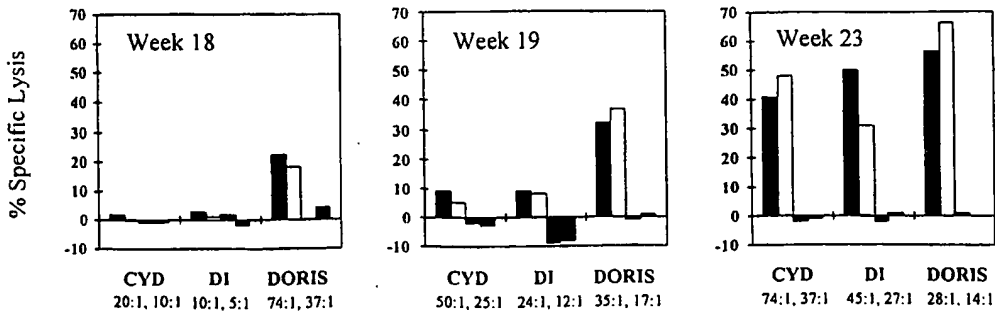


Figure 16



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: USE OF POXVIRUSES AS ENHANCER OF SPECIFIC IMMUNITY		
(57) Abstract The invention relates to a method for enhancing the specific immune response against an immunogenic compound which comprises administering the immunogenic compound together with a poxvirus recombinant and a vaccinal antigen, which is not a poxvirus. The immunological material may be any biological material useful as a vaccine <i>e.g.</i> , a polypeptide characteristic of a pathogenic microorganism or associated with a tumoral disorder, a DNA plasmid encoding a peptide or a polypeptide characteristic of a pathogenic microorganism or a tumor-associated antigen, or an hapten coupled to a carrier molecule. The poxvirus may be a live, attenuated or inactivated virus or a recombinant virus. Recombinant virus may encode a heterologous polypeptide such as chemokines, cytokines or co-immunostimulatory molecules or an homologous polypeptide, which is immunologically cross reactive with the immunogenic polypeptide or peptide.		

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Use of poxviruses as enhancer of specific immunity

The present invention relates to a method for enhancing the specific immune response against an immunogenic compound, which comprises administering the immunogenic
5 compound together with a poxvirus, recombinant or not.

Smallpox, a human infectious disease due to a vaccinia virus, was declared eradicated from the globe in 1980. This unique success was made possible by the availability of an effective virus-attenuated vaccine. Concurrent with the smallpox
10 eradication and the cessation of vaccination, a new use for the vaccinia virus was proposed (Panicali & Paoletti, PNAS (1982) 79: 4927). Utilizing molecular cloning techniques, it became possible to express genes from foreign pathogens in vaccinia virus providing new approaches to vaccination.

15 Since then, the original technology has been applied to the whole poxvirus family, including not only the vaccinia virus but also avipoxviruses such as fowlpox and canarypox. In order to address the issue of safety, a strategy was developed to genetically engineer a highly attenuated vaccinia virus such as the Copenhagen strain that would still retain the ability to induce vigorous immunological response against extrinsic antigens.
20 A number of poxvirus constructions have been tested in clinical trials. As a matter of example, they include recombinant vaccinia and canarypoxviruses expressing Human Immunodeficiency Virus (HIV) or *Plasmodium falciparum* antigens. Further, it has already been proposed to combine, in an immunization protocol, a prime-administration using a recombinant poxvirus vector and booster-administrations of the purified
25 polypeptide as encoded by the recombinant vector (See *e.g.*, Excler & Plotkin, AIDS (1997) 11 (suppl. A): S127). Such immunization protocols are commonly referred as prime-boost protocols and are very advantageous in a number of cases, in particular for AIDS treatment or prevention.

30 Prime-boost protocols are however unpractical both for physicians, manufacturers and sellers, in that they require two different pharmaceutical products that have to be each identified and licensed for their specific use (priming or boost).

It has now been found that poxvirus particles may be useful as enhancer of specific immunity. Indeed, it has been observed that the immune response against a vaccinal antigen, such as an HIV or an influenza virus protein, is enhanced, when it is
5 mixed with a poxvirus, recombinant or not. Additionally, It has also been found that an immunization protocol exclusively using a composition comprising a polypeptide and a poxvirus encoding this polypeptide, may be just as good as a prime-boost protocol. It has also surprisingly been found that the observed immunization effect is not a mere additional effect, but results from a synergism effect between the two components.

10

Therefore, the invention provides for:

- (i) The use of a poxvirus for the manufacture of a pharmaceutical composition comprising an immunogenic compound for inducing an immune response in a
15 vertebrate, wherein the poxvirus is able to enhance a specific immune response to the immunogenic compound.
- (ii) The use of a mixture comprising (a) an immunogenic compound which comprises at least one antigenic determinant characteristic of a pathogenic
20 microorganism or is cross-reactive with a tumor-associated antigen (TAA) and (b) a poxvirus; in the manufacture of a medicament to be administered to a vertebrate for treating or preventing an infection induced by the pathogenic microorganism or a tumoral disorder characterized by the malignant expression of the TAA; whereby said poxvirus enhances the specific immune response of the
25 vertebrate against said immunogenic compound.
- (iii) A pharmaceutical composition comprising (a) an immunogenic compound and (b) a poxvirus encoding an heterologous polypeptide which is selected from the
30 group consisting of adhesion molecules, co-immunostimulatory molecules, apoptotic factors, cytokines, chemokines and growth hormones.

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- (iv) A pharmaceutical composition comprising (a) an immunogenic compound which is a first polypeptide and (b) a poxvirus encoding an heterologous polypeptide, which has an amino acid sequence identical to the amino acid sequence of the first polypeptide.
- 5
- (v) A pharmaceutical composition comprising (a) an immunogenic compound which is a DNA plasmid encoding a first polypeptide and (b) a poxvirus encoding a second heterologous polypeptide, which has an amino acid sequence identical to the amino acid sequence of the first polypeptide.
- 10
- (vi) A method for enhancing the specific immune response of a vertebrate to an immunogenic compound, which comprises administering to the vertebrate the immunogenic compound together with a poxvirus, whereby the poxvirus enhances the specific immune response to the immunogenic compound.
- 15
- (vii) A method for treating or preventing in a vertebrate, a disorder either induced by a pathogenic microorganism or characterized by the malignant expression of a T.A.A, which comprises administering to the vertebrate, (a) an immunogenic compound which comprises at least one antigen determinant characteristic of the pathogenic microorganism or a tumor-associated antigen together with (b) a poxvirus; whereby a specific immune response to the immunogenic compound is induced in the vertebrate and whereby the poxvirus enhances the specific immune response.
- 20
- (viii) A method for enhancing the specific "in vitro" immunostimulation of cells from an immune system against a specific immunogenic compound, which comprises (a) recovering cells from a vertebrate, (b) "in vitro" incubating the cells with the immunogenic compound together with a poxvirus, whereby the cells are immunostimulated against the immunogenic compound and whereby the poxvirus enhances the immunostimulation and (c) administering the immunostimulated cells obtained from step (b) to a vertebrate.
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- 30

In a general manner, there exist two types of immunity: the innate immunity and the acquired immunity. The former which is phylogenetically older brings into play soluble molecules, *i.a.* complement factors and cells, such as NK cells or macrophages, which are innately programmed to detect noxious substances produced by pathogenic microorganisms and to provide for rapid but often incomplete antimicrobial host defense. The innate immune system intervenes as the first line of defense when an infectious agent attacks an individual. On the other hand, the innate immune system can not be educated by the antigens expressed by the pathogenic microorganisms or tumor cells during the life of an individual and in this respect, the innate immunity is confounded with the natural immunity. By contrast, the acquired immune system brings into play antigen-specific B and T lymphocyte clones the affinity of which increases by the time consecutively to repeated contacts with the specific antigen. Moreover, some of them behave as memory lymphocytes, since they have a long lasting life and are able to proliferate and expand rapidly consecutively to a further contact with a specific antigen, so that these memory lymphocytes contribute to the long term protection of an individual to infectious microorganisms. An essential goal of vaccination is to provide for these memory lymphocytes.

Accordingly, by "specific immune response" is meant a specific humoral and /or a specific cellular immune response against the immunogenic compound of the pharmaceutical composition. In the present invention, the specific humoral immune response includes both systemic and mucosal antibody responses since, to feature the humoral response, one may refer to all types of specific antibodies, *i.e.* IgM, all subclasses of IgG and IgA, that may be elicited by the pharmaceutical composition. The specific lymphoproliferative response and the specific cytotoxic T lymphocyte (CTL) response preferentially are the main parameters of the specific cellular immune response.

For use in the present invention, the immunogenic compound may be a chemical or a biological material that is able to induce a humoral or cellular immune response in a vertebrate. A biological material may be *e.g.*, an attenuated, inactivated or killed virus (to the exception of a poxvirus); a bacterial strain; a pseudovirion; a bacterial extract; a capsular polysaccharides; a peptide or a polypeptide found tumor-associated, cross-reactive with a TAA or characteristic of a pathogenic agent; or a DNA plasmid encoding

a peptide or a polypeptide as described above. As an example of chemical material, a hapten coupled to a carrier protein is cited.

By "hapten" is meant a molecule, generally of low molecular weight, which is
5 unable to trigger an antibody response by itself, but capable, after coupling with a carrier, to induce a specific antibody response which interacts specifically with the hapten molecule. For use in the present invention, such an hapten may be a peptide which amino acid sequence is at least 5 to 6 amino acid long (minimal size of an epitope) but of low molecular weight, a chemical molecule (such as dinitrophenol), or a drug. In a particular
10 embodiment of the present invention, a mixture according to the invention may be intended to treat drug addiction and to this end, may comprise a poxvirus, mixed with a drug, such as cocaine, coupled to a carrier molecule to induce an antibody response against the drug, in order to hamper both its fixation on the target cells, tissues or organs and the triggering of its narcotic effects. Methods of coupling a hapten to a carrier
15 molecule are of common use for a man skilled in the art.

By "polypeptide" or "protein" is meant any chain of amino acids, regardless of the length or post-translational modification (*e.g.*, glycosylation or phosphorylation). Both terms are used interchangeably in the present application.

20

Advantageously, immunogenic polypeptides may be polypeptides characteristic of a pathogenic microorganism *i.e.* a virus, bacteria or an eucaryotic parasite, or tumor-associated antigens (that are mammalian or avian antigens which are not normally expressed; their malignant expression is characteristic of a tumoral disorder) such as
25 tyrosinase, the MAGE protein family, the CEA, the ras protein, mutated or not, the p53 protein, mutated or not, Muc1, CEA and pSA.

For use in the present invention, immunogenic polypeptides may have amino acid sequences corresponding to the complete or partial sequence of naturally occurring
30 polypeptides. They may also have a sequence derived by amino acid deletion, addition or substitution from the naturally occurring sequences as far as they behave as immunologic equivalents *i.e.*, they are able to induce an immune response against the pathogenic microorganisms from which they derive or against the tumor. In other terms, an

immunogenic polypeptide is also meant to include any polypeptide that is immunologically cross-reactive with a naturally occurring polypeptide found in a pathogenic agent or tumor-associated.

- 5 By "immunologically cross-reactive polypeptides" is meant polypeptides that can be recognized by antibodies, *e.g.* polyclonal antibodies, raised against each of the polypeptides used separately, and advantageously in a substantially purified form.

As a matter of example, the polypeptide may be an HIV antigen such as the env, 10 gag, pol or nef protein. An HIV antigen is also meant to include any polypeptide that is immunologically cross-reactive with a naturally occurring HIV protein. For example, an HIV env protein may be the gp160 env precursor, or the gp120 or gp41 sub-unit. The gp160 precursor may be a soluble, non-cleavable precursor obtained by mutation of the cleavage site and deletion of the transmembrane region as described in U.S. Patent No 15 5,672,689. The precursor may also be truncated so that the C-terminal part of the gp41 region is removed (intracytoplasmic domain). The precursor may also be a hybrid precursor, combining in a single molecule, env sequences from various HIV strains. An HIV gag antigen may be the complete p55 precursor, the p13, p18 or p25 that naturally derive from p55, or any immunogenic gag protein fragment. In fact, a large variety of 20 polypeptides may be substituted for the naturally occurring HIV env, gag, pol or nef proteins, yet retaining their immunogenic properties.

As an additional example the polypeptide may be an influenza peptide or polypeptide which comprises the virus envelope components such as the haemagglutinin 25 and the neuraminidase and the virus internal components such as the protein M, the non-structural proteins and the nucleoprotein. An influenza peptide or polypeptide is also meant to include any precursor form of the mature envelope or internal proteins that are immunologically cross reactive with them. Likewise, the polypeptide or peptide may be any kind of haemagglutinin or neuraminidase of the influenza virus since there are 30 numerous antigenic variants of these two proteins.

For use in the present invention, the polypeptide characteristic of a pathogenic agent that is physically present in the composition may be purified from the pathogenic

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agent itself or recombinantly produced. Advantageously tumor-associated antigens (TAAs) as well will be produced by recombinant means. Standard expression vectors, promoters, terminators, etc and recombinant methods are now of common use for a man skilled in the art and recombinant expression can be readily achieved once an appropriate

5 DNA sequence corresponding to the polypeptide is available. In a particular embodiment, polypeptides may be recombinantly produced as fusion polypeptides (*i.e.*, a polypeptide fused through its N- or C- terminal end to any other polypeptide (hereinafter referred to as a peptide tail), using appropriate expression vectors, such as the pMal-c2 or pMal-p2 systems of New England Biolabs in which the peptide tail is a maltose

10 binding protein, or the His-Tag system available from Novagen.

An immunogenic compound, *e.g.*, a polypeptide physically present in a composition of the invention is advantageously present in a substantially purified form, *i.e.*, it is separated from the environment in which it naturally occurs and/or is free of the

15 majority of the polypeptides that are present in the environment in which it was synthesized.

As mentioned above, the immunogenic compound may also be a DNA plasmid unable to replicate in eucaryotic cells, comprising a DNA sequence encoding a peptide

20 or a polypeptide, this latter being defined as herein above, under the control of an appropriate promoter which allows the peptide or polypeptide to be expressed in eucaryotic cells after transfection by the recombinant plasmid. As a matter of example, the CMV (Cytomegalovirus) early promoter is broadly used for the expression of a heterologous peptide or polypeptide in human cells transfected with DNA plasmid

25 encoding peptide or polypeptide.

In a particular embodiment of the present invention, a DNA plasmid advantageously encodes a peptide comprising one or several epitopes characteristic of a viral, bacterial, parasitic, or tumor-associated polypeptide. As a matter of example, it is

30 well known that tumor-associated antigens, such as Her-2 neu, are often poor immunogens, because they are essentially "self" antigens. To overcome the lack of immunogenicity, it is commonly proposed to use as an immunogenic compound, instead of DNA encoding the whole polypeptide, a DNA encoding "subdominant" epitopes

selected from the polypeptide. This strategy is also applicable to infectious microorganisms, such as HIV, *Mycobacterium tuberculosis* or *Plasmodium falciparum* for which the protective antigens are not yet defined. In a particular embodiment of the invention, aimed at the induction or the enhancement of a specific CTL response in a variety of Major Histocompatibility Complex (MHC) contexts, a pharmaceutical composition comprising a poxvirus mixed together with a DNA plasmid encoding customized peptides, may be useful. A customized peptide comprises or mimics an epitope selected throughout the whole amino acid sequence of an antigen of a pathogenic micro-organism or a tumor, as containing putative anchor motifs needed for binding to various MHC class I molecules (such as in humans, HLA-A1, HLA-A2, HLA-B7,...). The customized peptides encoded by the plasmid may all together preferably trigger a specific CTL response in the main MHC contexts of a given vertebrate.

For use in the present invention, the poxvirus may be any virus belonging to the poxviridae family. Accordingly, useful poxviruses include, capripoxvirus, suipoxvirus, molluscipoxvirus, yatapoxvirus, entomopoxvirus, orthopoxvirus and avipoxvirus; these two latter being preferred. A typical orthopoxvirus is a vaccinia virus. A suitable vaccinia virus may be e.g., the highly attenuated Copenhagen strain or the NYVAC vector that is derived from the Copenhagen strain by precise deletion of 18 open reading frames (ORFs) from the viral genome as described in Tartaglia et al, Virology (1992) 188: 217. A typical avipoxvirus is a canarypoxvirus or a fowl poxvirus. A suitable canarypoxvirus may be e.g., the ALVAC vector obtained as described in Tartaglia et al (supra). A suitable fowlpox vector may be e.g., the TROVAC vector which is a plaque-cloned isolate derived from the FP-1 vaccine strain licensed for vaccination of 1 day old chicken (sold by Merial, Lyon, France) and described in Taylor et al, Vaccine (1988) 6: 497.

A poxvirus for use in the present invention may be a live, attenuated or inactivated virus. By "live virus" is meant a virus that is fully capable to reproduce its natural infectious cycle into sensitive cells, comprising virus entry, uncoating, gene expression, DNA replication, virus assembly, maturation and release. In a particular embodiment, a live virus may be attenuated. Attenuated virus may be obtained, e.g., by selection of spontaneous mutants after repeated infectious cycles into sensitive cells, by

selective pressure or deletion of non-essential genes using molecular biology tools. Nevertheless, whatever the process of attenuation, the viruses that are issued remain able to reproduce themselves into sensitive cells even if sometimes the spectrum of sensitive cells can decrease. As a matter of example, it may be useful to delete the vaccinia virus genome from K3L or E3L genes to render it more sensitive to the action of interferons and consequently to reduce its host restriction range (Beattie E and al., (1996) Virus Genes, 12, 89-94). As a matter of example a suitable live virus for use in humans may be a canarypoxvirus, since in human cells such a virus exhibits an abortive infectious cycle. Additionally a suitable attenuated virus for use in humans may be a NYVAC vector. By "inactivated virus" is meant a virus that is no more capable to reproduce its entire infectious cycle into sensitive cells as a result of either a mechanical, chemical or physical treatment. As may be easily understood, inactivation is particularly advantageous when a non-recombinant poxvirus is used.

For use in the present invention, a poxvirus may be recombinant or not. A non-recombinant poxvirus does not encode any heterologous polypeptide. On the other hand, a recombinant virus is typically a virus in the genome of which is inserted one or several foreign genes (*e.g.* an heterologous coding sequence located in the genome under the control of a viral promoter allowing at least a transient expression in the virus-infected cells).

A useful recombinant poxvirus encodes a heterologous peptide or polypeptide that may be of any kind. In one embodiment of the invention, the peptide or the polypeptide may be a cytokine, such as interleukin-2 (IL-2), interleukin-3 (IL-3) interleukin-12 (IL-12), interleukin-15 (IL-15), interleukin-18 (IL-18) and granulocyte macrophage-colony stimulating factor (GM-CSF); a chemokine, such as RANTES (Regulated on Activation Normal T-cell Expressed and Secreted) and MCP1 (Monocyte Chemotactic protein 1); a co-immunostimulatory molecule, such as B7, CD40, CD40L and ICAMs (inter cellular adhesion molecules); an adhesion molecule; an apoptotic factor, such as p53 and TNF (tumor necrosis factor); or an hormone such as a growth hormone.

In another embodiment, the immunogenic compound for use in the present invention is a peptide or a polypeptide and the admixed poxvirus encodes a heterologous peptide or polypeptide that cross-reacts with the immunogenic compound. Accordingly, the invention also features the use of a poxvirus for the manufacture of a pharmaceutical composition comprising a first polypeptide; wherein the poxvirus encodes a second polypeptide which immunologically cross reacts with the first polypeptide. The encoded polypeptide may be the same as the one present in the composition. In other words, the encoded polypeptide has an amino acid sequence identical to that of the polypeptide present in the composition. Alternatively, the poxvirus may encode an immunogenic polypeptide that is similar to the polypeptide present in the composition, although slightly different at the amino acid sequence level. In a particular embodiment, the immunogenic polypeptide present as such in the composition originates from a particular pathogenic strain and the poxvirus vector accompanying the polypeptide encodes an allelic variant thereof *i.e.*, the same polypeptide but from another strain. As a result, the polypeptide physically present and the encoded polypeptide may have amino acid sequences slightly different, being at least 70, 80, 90 % or more identical. A composition comprising the HIV MN gp120 together with a poxvirus encoding HIV LAI gp120 is cited as a matter of example. In another embodiment, the sequences of both the polypeptide physically present and the encoded polypeptide may derive from each other by addition, deletion or substitution of one or several amino acids, provided that these polypeptides are immunologically cross-reactive. As a matter of example, it is cited a composition comprising:

- (i) HIV gp160 and a poxvirus encoding HIV gp120;
- (ii) HIV gp160 in a soluble and non-cleavable form and a poxvirus encoding wild-type gp160;
- (iii) HIV gag p55 and a poxvirus encoding gag p18; or
- (iv) HIV gp120 and a poxvirus encoding HIV gp120-p18 hybrid protein; or
- (v) HIV gp120, HIV p18 and a poxvirus encoding HIV gp120-p18 hybrid protein.

As illustrated in section (v) hereinabove, a composition of the invention may comprise not only one but also two or more polypeptides present as such. The poxvirus may also encode several immunogenic polypeptides, at least one being immunologically cross-reactive with a polypeptide physically present in the composition; or the

composition may contain several poxviruses. Advantageously, when several polypeptides are present as such, the compositions of the invention further contain a poxvirus that operatively encodes polypeptides, each of them being two-by-two cross-reactive with the polypeptides physically present. Alternatively, the composition may
5 contain several poxviruses, each of them encoding a polypeptide cross-reactive with a polypeptide physically present. As understood by a man skilled in the art, a large variety of combinations are possible.

Recombinant pox vectors may be constructed using the basic two-step technique of
10 Piccini et al, (1987) in "Meth. In Enzymology" Acad. Press, San Diego and widely used for any pox vector as described in U.S. Patents Nos 4,769,330, 4,772,848, 4,603,112, 5,100,587 and 5,179,993. First, the heterologous DNA sequence to be inserted into the poxvirus is placed under the control of a suitable poxvirus promoter able to direct expression of the sequence in avian or mammalian cells. The expression cassette is then
15 introduced into an *E. coli* plasmid that contains a DNA region homologous to a non-essential locus of the pox vector DNA. The expression cassette is positioned so that it is flanked on both ends by poxvirus homologous DNA sequences. The resulting plasmid is then amplified by growth within *E. coli* and isolated. Second, the isolated plasmid containing the expression cassette to be inserted is transfected into a cell culture, *e.g.*
20 chick embryo fibroblasts, along with the poxvirus. Recombination between homologous poxvirus DNA present on the plasmid and the viral genome gives a recombinant poxvirus modified by the presence, in a non-essential region of its genome, of the expression cassette containing the heterologous DNA sequence.

25 For use in the present invention, poxviruses, irrespective of whether they are recombinant or not, may be propagated on mammalian cells such as Vero cells, BHK21 cells and Chick Embryo Fibroblasts (CEF), as described in *e.g.*, Piccini et al, and Taylor et al (*supra*). Once propagated, the viral particles may be merely harvested and clarified by centrifugation. They may also be purified further according to Joklick et al, Virology
30 (1962) 18: 9.

Compositions and/or methods of the invention are useful for both therapeutic and prophylactic purposes. When the immunogenic compound is characteristic of a

pathogenic microorganism or a T.A.A., the specific immune response induced upon administration of the compositions or resulting from the methods of the invention, is advantageously protective against the pathogenic microorganism or the tumoral disorder. As a matter of example, there is a need to improve the current influenza vaccine which is not optimally protective in old people. Such pharmaceutical compositions or methods of the invention provide for improved protection over the flu vaccine of the prior art as exemplified in example 6.

Compositions of the invention can be manufactured in a conventional manner. In particular, the compounds can be formulated with a pharmaceutically acceptable diluent or carrier *e.g.*, water or a saline solution such as phosphate buffer saline. In general, a diluent or carrier can be selected on the basis of the mode and route of administration, and standard pharmaceutical practice. Suitable pharmaceutical diluents or carriers as well as pharmaceutical necessities for their use in pharmaceutical formulations are described in *Remington's Pharmaceutical Sciences*, a standard reference text in this field.

A composition of the invention may be administered to any kind of vertebrate, *i.a.* to mammals or birds, in particular to humans. To this end, one can use any conventional route in use in the vaccine field *e.g.*, via parenteral routes such as the intravenous, intradermal, intramuscular and sub-cutaneous route or mucosal routes such as nasal or oral routes. Especially, for the immunotherapy of cancer it may be useful to administer the pharmaceutical composition intratumorally or into the neighbor lymph nodes.

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Compositions comprising a DNA plasmid as immunogenic compound, may advantageously be administered into the epidermis using a special device such as a gene gun or an equivalent device, or by intramuscular route. Taking into account that most of poxvirus are able to infect epidermis cells, it is worth noticing that the composition of the invention and advantageously a composition comprising a DNA plasmid mixed with a poxvirus is suitable for an intradermal or transcutaneous immunization as described by Glenn GM et al, (1998), J. Immunol. 161: 3211-3214.

In a general manner, the administration can be achieved in a single dose or repeated at intervals, *e.g.* repeated twice or more, one or two months apart.

In compositions of the invention, the appropriate dosage of the poxvirus, and the immunogenic compound depends on various parameters understood by skilled artisans such as the vector and the immunogenic compound themselves, the route of administration, the general status of the vertebrate to be vaccinated (weight, age and the like), the type of immune response that is desired and the tumoral or infectious site. An efficient amount of the compounds is such that upon administration, an immune response against the compounds will be induced. For guidance, it is however indicated that the infectious titer (amount of virus able to infect 50 % of a cell culture) per dose of the poxvirus may suitably range from 10^3 to 10^9 , preferably from 10^5 to 10^8 CCID₅₀ (Cell Culture Infectious Dose 50). The polypeptide(s) physically present in the composition may amount from 10 µg to 1 mg, advantageously from 25 to 500 µg, preferably from 50 to 200 µg ; most preferably, a single dose contains about 50-100 µg of polypeptide(s). Whenever a DNA plasmid is the immunogenic compound, a convenient dose of DNA plasmid administered may amount from several ng to a few mg depending on the size of the animal giving the composition. In human beings the suitable dose of DNA plasmid per immunization may range from 20µg to 2500µg as mentioned by Wang R et al (1998), Science, 282, 476-480

All the documents cited throughout the specification are incorporated by reference.

The invention is further explained and illustrated in the examples by reference to the figures described as follows.

Figures 1a and 1b refer to Example 1 and show mean gp160 MN/LAI ELISA antibody titers (log) in guinea-pigs immunized twice by intramuscular route (on days 1 and 29) with vCP205 and/or gp160 MN/LAI 4 µg (1a) or 40 µg (1b).

Figures 2a and 2b refer to Example 1 and show mean V3 MN ELISA antibody titers (log) in guinea-pigs immunized twice by intramuscular route (on days 1 and 29) with vCP205 and/or gp160 MN/LAI 4 µg (2a) or 40 µg (2b).

In Figures 1a and 2a : ○ corresponds to group #1 (D1 and D29 : gp160) ; ● corresponds to group #3 (D1 : vCP205 and D29 : gp160) ; ∇ corresponds to group #5 (D1 and D29 : vCP205 + gp160) ; and ▼ corresponds to group #7 (D1 and D29 : vCP205).

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In Figures 1b and 2b : ○ corresponds to group #2 (D1 and D29 : gp160) ; ● corresponds to group #4 (D1 : vCP205 and D29 : gp160) ; ∇ corresponds to group #6 (D1 and D29 : vCP205 + gp160) ; and ▼ corresponds to group #7 (D1 and D29 : vCP205).

10 Figure 3 refers to Example 2 and shows CPpp antibody titers (log/ml) in guinea-pigs inoculated twice intramuscularly with various doses of vCP205. ● corresponds to group #3 ($10^{4.8}$ CCID₅₀) ; ▼ corresponds to group #5 ($10^{5.8}$ CCID₅₀) ; and ■ corresponds to group #8 ($10^{6.1}$ CCID₅₀).

15 Figures 4a and 4b refer to Example 2 and show gp160 MN/LAI ELISA antibody titers (log/ml) in guinea-pigs inoculated twice intramuscularly with various doses of vCP205 and/or gp160 MN/LAI.

In Figure 4a, ● corresponds to group #1 (40 µg of gp160), ▼ corresponds to group #2 (80 µg of gp160) ; ■ corresponds to group #4 ($10^{4.8}$ CCID₅₀ of vCP205 + 40 µg of gp160) ; ▲ corresponds to group #6 ($10^{5.8}$ CCID₅₀ of vCP205 + 40 µg of gp160 mixed together) ; and ◆ corresponds to group #7 ($10^{5.8}$ CCID₅₀ of vCP205 + 40 µg of gp160 injected separately).

20
25 In Figure 4b, ○ corresponds to group #3 ($10^{4.8}$ CCID₅₀ of vCP205), ∇ corresponds to group #5 ($10^{5.8}$ CCID₅₀ of vCP205), □ corresponds to group #8 ($10^{6.1}$ CCID₅₀ of vCP205), ■ corresponds to group #4 ($10^{4.8}$ CCID₅₀ of vCP205 + 40 µg of gp160), ▲ corresponds to group #6 ($10^{5.8}$ CCID₅₀ of vCP205 + 40 µg of gp160 mixed together), ◆ corresponds to group #7 ($10^{5.8}$ CCID₅₀ of vCP205 + 40 µg of gp160 injected separately).
30

Figures 5 to 8 refer to Example 3 and show the mean ELISA antibody titers (log/ml) in macaques immunized intramuscularly with $10^{6.5}$ CCID₅₀ vCP205 and/or 100 µg gp160

MN/LAI adjuvanted or not (Figure 5 : gp160 ELISA antibody ; Figure 6 : V3 MN ELISA antibody ; Figure 7 : p24 LAI ELISA antibody ; Figure 8: CPpp ELISA antibody). ♦ corresponds to group #1 ; ∇ corresponds to group #2 ; ○ corresponds to group #3 ; and ▼ corresponds to group #4. (■ is irrelevant).

5

Figure 9 refers to Example 3 and shows the HIV MN seroneutralizing antibody titers (log) in macaques immunized five times intramuscularly with $10^{6.5}$ CCID50 vCP205 and/or 100 µg gp160 MN/LAI adjuvanted or not at weeks 0 (square-dotted box), 16 (hatched box) and 26 dotted box). Schemes A to D correspond respectively to groups #1 to #4.

10

Figures 10a and 10b refer to Example 4 and show ELISA CPpp antibody (1a) and gp160 MN/LAI antibody (1b) mean titers in guinea-pigs primed intramuscularly with a mixture of gp160 MN/LAI (5µg) and different doses of crude or purified CPpp, then boosted with 5 µg of gp160 MN/LAI. ○ corresponds to group #1 ; ● corresponds to group #2 ; ∇ corresponds to group #3 ; ▼ corresponds to group #4 ; and □ corresponds to group #5.

15

Figures 11a and 11b refer to Example 5 and show ELISA IgG CPpp antibody (2a) and gp160 MN/LAI antibody (2b) mean titers in guinea-pigs primed intramuscularly with a mixture of gp160 MN/LAI (5 µg) and different fractions of ALVAC-Luc (vCP297), either inactivated or not, then boosted (week 4) with 5 µg of gp160 MN/LAI. ○ corresponds to group #1 ; ● corresponds to group #2 ; ∇ corresponds to group #3 ; ▼ corresponds to group #4 ; and □ corresponds to group #5.

20

Figures 12 and 13 refer to example 6 and show respectively the IgG1 and IgG2a ELISA antibody titers specific for A/Texas in each individual aged DBA/2 mice immunized twice with either 3µg of A/Texas (group 1), 2×10^7 CCID50 of CPpp and 3µg of A/Texas (group 2) or 2×10^7 CCID50 of CPpp (group 3). ■ and ♦ correspond to mice of group 1 respectively after one and two immunizations. ▲ and □ correspond to mice of group 2 respectively after one and two immunizations. ◆ and ◇ correspond to mice of group 3 respectively after one and two immunizations.

25

30

Figure 14 refers to example 6 and shows the survival curves of the 3 immunized groups after a lethal challenge with A/Taiwan. Δ corresponds to group 1; \square corresponds to group 2; \blacklozenge corresponds to group 3.

- 5 Figure 15 refers to example 6 and shows the morbidity curves of the 3 immunized groups after a lethal challenge with A/Taiwan. Δ corresponds to group 1; \square corresponds to group 2; \blacklozenge corresponds to group 3.

10

- 15 Example 1: Simultaneous immunization with ALVAC-HIV (vCP205) and gp160 MN/LAI in guinea pigs

1A - vCP205 preparation

- 20 vCP205, an ALVAC pox vector capable of expressing HIV proteins, is described in Example 14 of WO 95/27507. Briefly, it contains a first heterologous sequence encoding the env gp120 MN + the transmembrane region of LAI gp41, and a second sequence encoding LAI (gag + protease); these sequences are inserted in the C3 locus and placed under the control of promoters H6 and I3L.

25

Clarified vCP205 was produced on chick embryo fibroblasts in DMEM - Ham F12 medium without serum, harvested in lactoglutamate and clarified by centrifugation.

The preparation used hereinafter has a mean titer of $10^{8.5}$ CCID₅₀ / ml on QT35 cells.

- 30 Purified vCP205 was produced as described above and further purified according to Joklick et al, (supra). The vCP205 preparation in phosphate buffer saline (PBS) 20 mM pH 7.2 (in the absence of Mg^{++} and Ca^{++}) as used hereinafter, has a mean titer of $10^{8.8}$ CCID₅₀ / ml on QT35 cells.

1B - gp160 MN/LAI preparation

A recombinant vaccinia virus vector, VVTG9150, is used for gp160 production. VVTG9150 operatively encodes a hybrid, soluble HIV-1 gp160 in which the gp120 moiety derives from HIV-1 MN and the gp41 trans-membrane part comes from the LAI isolate. The DNA sequences corresponding to these two compounds are fused together using an artificial *Sma*I restriction site, which modifies neither the gp120, nor the gp41 amino acid sequence. The construction of the two partners is briefly described as follows.

The sequence encoding the MN gp120 was amplified from cells SupT1 infected with HIV- MN, using the PCR technique with oligonucleotides which introduce a *Sph*I and *Sma*I restriction sites respectively located immediately downstream of the sequence encoding the leader peptide and upstream of the cleavage sites located between gp120 and gp41.

The sequence encoding the LAI gp41 was produced as follows: The complete HIV-1 LAI env coding sequence was placed under the control of the vaccinia pH5R promoter. Several modifications were introduced into this encoding sequence. First a *Sph*I restriction site was created immediately downstream of the sequence encoding the leader peptide, without altering the amino acid sequence. Second, a *Sma*I restriction site was created immediately upstream of the sequence encoding the cleavage sites between gp120 and gp41, without altering the amino acid sequence. Third, the two cleavage sites in position 507 – 516 (amino acids numbered according to Myers et al, In: Human retroviruses and AIDS (1994) Los Alamos National Lab. (USA)) were mutated (original sequence KRR ... REKR mutated into QNH ... QEHN). Fourth, the sequence encoding the transmembrane hydrophobic peptide IFIMIVGGLVGLRIVFAVLSIV (amino acids 689 – 710 in Myers et al (supra)) was deleted. Fifth, a stop codon was substituted for the second E codon of the sequence encoding PEGIEE (amino acids 735 – 740 in Myers et al (supra)) *i.e.*, the 29th amino acid of the intracytoplasmic domain.

The plasmid, in which the LAI sequence was inserted between vaccinia virus thymidine kinase (TK) gene homologous regions, was cut with *SphI* and *SmaI* and further ligated with the MN gp120 sequence. VVTG9150 was then constructed by conventional homologous recombination and propagated for MN/LAI gp160 expression according to the conventional method used for vCP205 on BHK21 cells. The protein was purified by immunoaffinity chromatography.

1C - Experimental procedure

10 Guinea pigs were submitted to immunization protocols as described in Table 1 hereinafter.

Table 1

Group # (Guinea-pig)	Inoculation days	
	D1	D29
1 (1, 2, 3, 4, 5)	4 µg gp160	4 µg gp160
2 (6, 7, 8, 9, 10)	40 µg gp160	40 µg gp160
3 (11, 12, 13, 14, 15)	10 ^{6.1} CCID50 ALVAC-HIV	4 µg gp160
4 (16, 17, 18, 19, 20)	10 ^{6.1} CCID50 ALVAC-HIV	40 µg gp160
5 (21, 22, 23, 24, 25)	10 ^{6.1} CCID50 ALVAC-HIV + 4 µg gp160	10 ^{6.1} CCID50 ALVAC-HIV + 4 µg gp160
6 (26, 27, 28, 29, 30)	10 ^{6.1} CCID50 ALVAC-HIV + 40 µg gp160	10 ^{6.1} CCID50 ALVAC-HIV + 40 µg gp160
7 (31, 32, 33, 34, 35)	10 ^{6.1} CCID50 ALVAC-HIV	10 ^{6.1} CCID50 ALVAC-HIV

15

Each dose was administered intramuscularly under a final volume of 1.2 ml (0.6 ml in each thigh). When vCP205 and gp160 were both administered, these two products were mixed together before.

20 Serological analyses were carried out with blood samples collected on days 0 (one day before the first immunization), 28, 43 and 57. Antibodies to HIV gp160 glycoprotein and V3 peptide were titrated by ELISA as follows:

Maxisorp F96 NUNC plates were coated for 1 hour at 37°C, then overnight at 4°C, with one of the following antigens, diluted in 0.1 M carbonate buffer, pH 9.6: 130 ng per well of purified gp160 MN/LAI; 200 ng of V3 peptide from HIV MN.

5

Plates were then blocked for 1 hour at 37°C with 150 µl of phosphate buffered saline (PBS) pH 7.1 - 0.1 % Tween 20 - 5 % (w/v) powdered skim milk, (PBS-Tween-milk). All next incubations were carried out in a final volume of 100 µl, followed by 3 or 4 washings with PBS, pH 7.1 - 0.1 % Tween 20.

10

Serial threefold dilutions of the sera, ranging from 1/100 to 1/24300 or 1/1000 to 1/243000, in PBS-Tween-milk, were added to the wells and incubated for 90 min at 37°C. After washings (3 times), anti-guinea-pig IgG peroxylase conjugate (Sigma, rabbit IgG fraction) was diluted at 1/3000 in PBS-Tween-milk, added to the plates and incubated for another 90 min at 37°C. The plates were further washed (4 times) and incubated in the dark for 30 min at room temperature with O-phenylenediamine dihydrochloride (Sigma) at 1.5 mg/ml in 0.05 M phosphate citrate buffer, pH 5.0 containing 0.03 % sodium perborate (Sigma). The reactions were stopped with 50 µl of 4N H₂SO₄.

20

The optical density (OD) was measured at 490-650 nm with an automatic plate reader (Vmax, Molecular Devices). The blanks (mean value) were subtracted to the data and duplicate values averaged. The antibody titers were calculated for the OD value range of 0.2 to 1.3, from the regression curve of a standard hyperimmune guinea-pig serum specific for both gp160 and V3 antigens, present on each ELISA plate.

25

The titer of the standard serum had been previously determined according to the formula:

$$\text{Titer} = \log \frac{\text{OD}_{490-650} \times 10 (\text{OD value range: 0.2 to 1.3})}{1 / \text{dilution}}$$

30

1D - Serological results

Averaged titers for each group of guinea pigs are presented in Figures 1 (gp160 antibody titers) and 2 (V3 antibody titers).

Comparison of the anti-HIV antibody responses induced by gp160 alone (groups #1 and #2), vCP205 alone (group #7), and combination of both antigens (groups #5 and #6)

Antibody responses to gp160

The lowest responses were observed, after both the primary and booster immunizations, in guinea pigs that received 4 µg of gp160 (group #1). With 40 µg of gp160 (group #2), humoral responses were much more elevated: only one inoculation was required for all animals to seroconvert, *versus* two with the 4 µg dose ; and the mean antibody titers to V3 and gp160 were higher in group #2 than in group #1 (> +1 log higher on week 6).

vCP205 ($10^{6.1}$ CCID₅₀) injected alone (group #7) elicited anti-HIV antibodies at comparable but lower levels than those induced by gp160 alone at 40 µg, especially after the booster injection (difference in mean titers \approx -0.4 log on week 6).

Mixing vCP205 with 4 µg of gp160 (group #5) was not found to significantly enhance the antibody response comparatively to vCP205 alone. Conversely, and of great interest, two immunizations with the combination vCP205 plus gp160 at 40 µg (group #6) induced the best antibody titers, higher than those raised by vCP205 alone (group #7) (raise of mean ELISA titers \approx +0.8 log on week 6) and, in lesser extent, by 40 µg of gp160 alone (group #2) (\approx +0.4 log on week 6).

Antibody responses to V3

Although the antibody titers raised against the V3 domain were, as previously observed, lower than those induced against whole gp160, the reactivity pattern to V3 was similar to that obtained to gp160. In particular, the (vCP205 plus 40 µg of gp160) combination proved to be the best immunogen, whereas the 4 µg dose of gp160 injected alone was the worst.

Comparison of the anti-HIV antibody responses induced by the mixture of vCP205 plus gp160 (groups #5 and #6) and by a prime (vCP205) / boost (gp160) immunization regimen (groups #3 and #4)

- 5 As observed in previous tests, a clear priming effect of vCP205 on the anti-HIV humoral responses following a boost with gp160 (at either 4 or 40 µg) was found. Nonetheless, animals immunized according to this prime/boost regimen displayed lower responses to V3 than those inoculated with two injections of the mixture vCP205 plus gp160 (using 4 or 40 µg of gp160). Similar differences were seen when anti-gp160 responses were
10 considered, but only with 40 µg of gp160.

- Noticeably, the prime/boost immunization using: (i) 40 µg of gp160 (group #4) gave antibody levels equivalent to those elicited by two inoculations of gp160 alone at 40 µg (group #2); or (ii) 4 µg of GP160 (group #3) raised antibody titers similar to or lower
15 than those induced by two injections of vCP205 (group #7).

General conclusion

- Immunogenicity of the different combinations of ALVAC-HIV vCP205 and/or gp160
20 MN/LAI evaluated in the present study in guinea pigs can be classified as followed:

$$\text{gp160 (4 µg)} < \text{prime vCP205 / boost gp160 (4 µg)} = \text{vCP205} = \text{vCP205 + gp160 (4 µg)}$$
$$= \text{prime vCP205 / boost gp160 (40 µg)} = \text{gp160 (40 µg)} < \text{vCP205 + gp160 (40 µg)}.$$

- 25 In particular, these results revealed that two co-injections of vCP205 and gp160 can induce higher anti-HIV serological responses (to V3 and gp160) than two inoculations of either vCP205 or gp160 alone, or than a prime (vCP205) / boost (gp160) immunization. Such an enhancing effect was observed mainly when vCP205 was combined with a high dose of gp160 (40 µg) but not with a lower one (4 µg).

- 22 -

Example 2: Analysis of the enhancing effect of a mixture vCP205 + gp160 MN/LAI on the antibody response to gp160 MN/LAI in guinea-pigs

The experiment reported in Example 2 were performed in guinea-pigs (i) to confirm the ability of the mixture gp160 MN/LAI plus vCP205 to stimulate the antibody response to gp160, as previously observed in Example 1; (ii) to determine whether this enhancement results from a simple additive or rather a synergistic effect between the two immunogens; and (iii) to evaluate whether such an effect can be obtained when the two products are inoculated simultaneously at distinct sites or only when they are mixed.

10

2A - vCP205 preparation was achieved as described in Example 1A hereinabove

2B - gp160 preparation was achieved as described in Example 1B hereinabove

15 2C - Experimental procedures

Thirty-nine guinea pigs distributed in eight groups received vCP205 and/or gp160 doses as stated in Table 2.

20 Table 2

		ALVAC-HIV (vCP205) (CCID50)				
		0	$10^{4.8}$	$10^{5.8}$		$10^{6.1}$
gp160 (μ g)	0		# 11 to 14	# 21 to 24		# 36 to 40
	40	# 1 to 5	mixed # 15 to 19	mixed # 25 to 30	separately # 31 to 35	
	80	# 6 to 10				

Each guinea pig received intramuscularly two identical injections (each under a volume of 1.2 ml), one month apart. The viral vector and the mixtures were administered in both thighs, whereas gp160 alone was administered in the right fore leg.

25

Serological analyses were carried out with blood samples collected on days 1, 15, 28, 43 and 57. Antibodies to HIV gp160 MN/LAI glycoprotein and to non-recombinant purified canary pox (CPpp) were titrated by ELISA as described in Example 1C. To this end, 500

ng of CPpp / well were used as well as a standard hyperimmune guinea-pig serum for CPpp.

2D - Serological results

5

Anti-CPpp antibody response

The antibody response elicited against CPpp was measured in the three groups of guinea pigs inoculated with $10^{4.8}$, $10^{5.8}$ or $10^{6.1}$ CCID50 of ALVAC-HIV (vCP205) alone
10 (groups #3, #5 and #8, respectively). The mean titers of each group are presented in Figure 3.

The doses of $10^{4.8}$ and $10^{5.8}$ CCID50 of vCP205 raised similar anti-CPpp antibody levels, which proved to be lower than those induced by the dose of $10^{6.1}$ CCID50 of
15 ALVAC-HIV, mostly after the first injection (difference in mean titers of ~ -0.7 log on week 4).

Anti-gp160 MN/LAI antibody response

20 The antibody response to gp160 MN/LAI was measured in all immunized animals. The mean titers of each group are represented in Figures 4a and 4b.

When the groups of guinea pigs were globally compared by variance analysis, a significant difference between immunogens was observed in the antibody response
25 elicited against gp160 ($p < 0.0005$).

Injections of either gp160 MN/LAI at 40 or 80 μ g (groups #1 and #2) or ALVAC-HIV (vCP205) at $10^{5.8}$ or $10^{6.1}$ CCID50 (groups #5 and #8) were found to induce close anti-gp160 antibody levels which proved to be statistically identical along the study.
30 ALVAC-HIV (vCP205) at the dose of $10^{4.8}$ CCID50 (group #3) appeared to raise lower antibody responses, the difference in mean titers with groups #1, #2, #5 and #8 ranging from -0.4 to 1.8 log during the serology, but statistical significance was evidenced only with group #8.

These results suggested that the gp160-specific humoral response elicited by the HIV protein at 40 to 80 µg or the recombinant ALVAC-HIV (vCP205) at $10^{5.8}$ or $10^{6.1}$ CCID50 had reached its maximum. However, mixture $10^{4.8}$ CCID50 vCP205 plus 40 µg gp160 (group #4) was found to induce elevated antibody titers which proved to be significantly higher than those raised (i) by vCP205 alone at $10^{4.8}$, $10^{5.8}$ or $10^{6.1}$ CCID50 (difference in mean titers ranging from +0.5 to +2.6 log), and (ii) by gp160 alone at 40 or 80 µg (difference in mean titers ranging from +0.8 to +2.5 log).

10 The anti-gp160 antibody levels induced by the mixture vCP205 at $10^{5.8}$ CCID50 plus 40 µg gp160 (group #6) also appeared to be high and did not significantly differ from those elicited in group #4 (mixture with vCP205 at $10^{4.8}$ CCID50). Moreover, the simultaneous injection of $10^{5.8}$ CCID50 vCP205 and 40 µg gp160 either mixed (group #6) or injected separately (group #7) gave similar increased antibody responses, as confirmed statistically.

Whether or not the strongest anti-gp160 antibody responses observed with the three combinations of vCP205 and gp160 (groups #4, #6 and #7) resulted from a simple additive or rather a synergistic effect between both immunogens was difficult to assess.

20 In an attempt to address this issue, the mean ELISA titers measured experimentally for each combination were compared to the estimated titers that would result from an additive effect between gp160 and vCP205. As shown in Table 4, the titers measured for the mixture with vCP205 at $10^{4.8}$ CCID50 (group #4) were found to be higher than the theoretical additive titers, the ratio "measured titer / theoretical additive titer" ranging from 5.4 to 165.5 along the serology. This ratio was also above 1 albeit never exceeding 10, for the group receiving the mixture with vCP205 at $10^{5.8}$ CCID50 (group #6). This was also true when gp160 was administered separately to vCP205 at the same dose (group #7), but only after the primo immunization (weeks 2 and 4).

30 These results suggested that a synergism between ALVAC-HIV (vCP205) and gp160, potentiating the antibody response to gp160, can occur. Such an effect would also take place when both immunogens are injected separately, although apparently less efficiently.

General conclusion

- The ability of the combination of gp160 MN/LAI (40 µg) and ALVAC-HIV (vCP205) (10^{4.8} or 10^{5.8} CCID50) to stimulate the humoral response to gp160 MN/LAI in guinea pigs was confirmed. The antibody levels elicited against the HIV protein by these mixtures were indeed increased comparatively to those obtained by each immunogen at either a similar or a two-fold (or more) higher dose (*i.e.* gp160 at 40 or 80 µg or ALVAC-HIV at 10^{4.8}, 10^{5.8} or 10^{6.1} CCID50).
- 10 This stimulating effect seemed to result from a synergistic rather than an additive phenomenon, and could also occur at distance when both antigens were injected at distinct sites.

- Example 3: Comparison of the immune response induced in rhesus macaques either
15 by a mixture of vCP205 + gp160 MN/LAI or a prime boost immunization
vCP205 / gp160 MN/LAI in aluminum hydroxide Al (OH)₃ (Alum)

- 3A - vCP205 preparation was achieved as described in Example 1A hereinabove
- 20 3B - gp160 preparation was achieved as described in Example 1B hereinabove
- 3C - Experimental procedure

- Thirteen rhesus macaques (*Macaca mulatta*) were immunized according to the
25 immunization protocols as shown in Table 3.

30

35

Table 3

Macaques		Immunizations (Weeks)				
Group #	Sex and number	W0	W4	W8	W12	W24
1	F1, F2	gp160	gp160	gp160	gp160	gp160
2	F4, F5, M6	gp160 + alum	gp160 + alum	gp160 + alum	gp160 + alum	gp160 + alum
3	M11, F12, M13, F18	ALVAC-HIV + gp160	ALVAC-HIV + gp160	ALVAC-HIV + gp160	ALVAC-HIV + gp160	ALVAC-HIV + gp160
4	F19, F20, F21, M22	ALVAC-HIV	ALVAC-HIV	gp160 + alum	gp160 + alum	gp160 + alum

F: female; M: male.

- 5 Macaques were administered doses intramuscularly in one thigh (right or left alternatively), under a final volume of 1 ml, comprising $10^{6.5}$ CCID₅₀ vCP205, 100 µg gp160 and/or 0.3 mg alum.

10 Blood samples were collected every two weeks, starting on week 0 (first immunization week).

Antibodies to HIV gp160 MN/LAI glycoprotein, V3 MN peptide, p24 LAI and CPpp were titrated by ELISA (Figures 5 to 8) as described in Example 1C. Reagent dosages were as follows: gp160 MN/LAI: 130 ng / well; V3 MN peptide: 200 ng / well; p24 LAI: 130 ng / well; and CPpp: 500 ng / well.

Two different peroxylase conjugates were used, diluted in PBS-Tween-milk, depending on the coating antigen:

- for the gp160 MN/LAI, V3 MN and p24 LAI titrations: goat anti-monkey IgG peroxylase conjugate (Cappel, ref. 55432) at 1/1,000
- for the CPpp titrations: sheep anti-human Ig peroxylase conjugate (Amersham, ref. NA 933) at 1/300.

Antibody titers were calculated for the OD value range of 0.2 to 1.3, from the regression curve of a standard specific hyperimmune macaque serum present on each ELISA plate.

Neutralizing test were also carried out (Figure 9). The assay determines the dilution of serum that prevents the development of syncytia in 50 % of microwells infected with 10 CCID₅₀ of HIV MN. The MN strain was obtained from F. Barré-Sinoussi and propagated in CEM clone 166 cells.

Sera were decompemented and twofold serial dilutions in RPMI beginning 1/10 were prepared. Equal volumes of serum dilution and HIV suspension (500 µl each) were mixed and incubated for 2 hrs at 37°C. The HIV suspension had been adjusted to contain 10² to 10^{2.5} CCID₅₀ per ml.

Prior to use, indicator CEMss cells were plated in microwells coated with poly-L-lysine, and incubated for 1 hr at 37°C. Culture medium was removed and replaced with the virus / serum mixtures (100 µl / well, 6 wells per dilution). After 1 hr incubation at 37°C, culture medium was added to each well and the plates were incubated at 37°C. All incubations were done in a 5 % CO₂ incubator.

After 7 and 14 days respectively, the cultures were examined under the microscope and wells showing syncytia were recorded. Neutralizing 50 % titer was computed according to SPEARMAN and KÄRBER and expressed as the log₁₀ of the end-point. As a confirmation, supernatants of the cultures were collected on day seven, pooled for each dilution and assayed for reverse transcriptase (RT) activity.

Each assay included a set of uninfected microwells as negative controls, an infectivity titration of the virus suspension and a titration of antibody in a reference serum.

3D - Serological results

The mean antibody kinetics are presented in Figures 5 to 9.

gp160 MN/LAI antibodies

All animals injected with gp160 MN/LAI only (group #1) seroconverted, although weakly, to the HIV protein after one immunization and consistently increased their response after the second and third inoculations (mean titers raised by +0.8 to +1.0 log two weeks post-injection). After the fourth immunization, titers reached similar levels than after the third one, and then decreased. The last inoculation induced a strong booster effect (mean titers raised by +1.3 log two weeks post-injection) and elicited the highest titers of the period examined (5.0 log on week 26).

10

A marked adjuvant effect of alum (group #2) was observed on the anti-gp160 antibody response in naive macaques. Indeed, as compared to the non-adjuvanted group (#1), the mean ELISA titers were enhanced by +1.0 to +2.0 log after each of the four first inoculations, and to a lesser extent after the fifth injection (+0.3 to +0.5 log). The highest levels of gp160-specific antibodies were obtained earlier than in group #1. This adjuvant effect was found to be significant (statistical analysis performed when possible, *i.e.* on weeks 4, 6 and 8, using the Dunnett's t-test).

Interestingly, the mixture (ALVAC-HIV+gp160) (group #3) was found to induce a significant higher response to gp160 than ALVAC-HIV after one or two inoculation(s) (group # 4) (difference in mean titers up to +1.5 log). The anti-gp160 antibody titers were also more elevated in macaques injected with the mixture than in the vCP205-primed animals boosted with gp160 in alum (group #4). However, the differences were slight (+ 0.7 log maximum) and found to be significant only on weeks 20, 24 and 28 (group #4) (Newman-Keuls test).

The combination (ALVAC-HIV+gp160) also proved to be a better immunogen than gp160 alone (group #1) (mean titers between +0.8 to +1.7 log higher along the experiment), and did not strongly differ from gp160 adjuvanted in alum (group #2) (differences in mean titers = +/- 0.5 log).

Finally, the prime/boost immunization regimen (group #4) induced in most cases higher antibody titers than inoculation with gp160 alone (group #1), especially after the gp160

boosts (differences up to +1.4 log), but lower responses than injection with gp160 in alum (group #2), particularly after the ALVAC priming (differences up to -2.0 log).

V3 MN antibodies

5

On the whole, antibody responses elicited against V3MN shew a similar pattern than against gp160MN/LAI, although to a lesser magnitude.

10 Alum (group #2) also increased the antibody titers to V3MN as compared to the non-adjuvanted group (#1), and this enhancing effect was found to be significant at weeks #2, 4, 6, 8.

15 Animals injected with the mixture (ALVAC-HIV+gp160) (group #3) displayed significantly increased anti-V3MN responses than those receiving the prime/boost immunization (group #4) but only after the first and the second priming with ALVAC-HIV (weeks 4, 6 and 8) and following the last gp160 boost (weeks 26 and 28) (Newman-Keuls test). Moreover, similarly to what was seen on gp160, and although no statistical analysis could be performed given the low number of animals tested, the mixture raised
20 V3MN responses higher than did gp160 alone (group #1) (titers augmented by +1.0 to +1.8 log), and close to those induced by gp160 adjuvanted in alum (group #2) (titers = +/- 0.5 log in most cases).

p24 LAI antibodies

25 In the group of macaques injected with the mixture (ALVAC-HIV+gp160) (#4), 2 animals out of 4 developed an antibody response against p24 LAI as compared to the preimmune samples: #11 became positive after two inoculations and titers increased by up to +1.3 log following the next immunizations; #18 clearly seroconverted after the third injection and maintained or decreased its response afterwards.

30

In group #5 receiving the prime/boost immunization, only 1 or possibly 2 from group #5 was (were) found to be positive on p24 LAI: #19 raised antibodies as soon as the first ALVAC priming; #22 was hardly positive after the last gp160 boost.

Anti-canarypox (CPpp) antibodies

All macaques immunized against ALVAC-HIV vCP205 either two (group #4) or five (group #3) times elicited CPpp-specific antibodies two weeks after the first injection and
5 reached their maximal responses after the second inoculation (week 6). Following the gp160 boosts in group #4, the anti-CPpp titers gradually decreased and were reduced by -1.0 log on week 28. In group #3, the mean antibody levels were maintained until week 14 (two weeks after the fourth injection), diminished (-0.7 log), and then increased to their maximum after the last booster immunization (week 26).

10

3E - HIV-1 MN neutralizing antibody response

The mean titers of each group of macaques are presented in Figure 9.

15 All the tested animals developed anti-HIV-1 MN neutralizing antibodies when examined after the fourth (week 16) and the fifth (week 26) injection, as compared to the preimmune samples (week 0).

Because of the low number of macaques studied in groups #1 and #2, no statistical
20 comparison could be performed for these animals. However, the lowest neutralizing titers were observed in group #1 inoculated with non-adjuvanted gp160. In group #2 (except for week 26), injected with gp160 adjuvanted in alum, the neutralizing response was stronger than in group #1, similar on week 16 and higher on week 26 than in group #4 (prime/boost immunization), and slightly lower than in group #3 injected with the
25 (ALVAC-HIV+gp160) mixture.

Paired comparisons of groups #3 and #4 by the Newman-Keuls test revealed no statistical difference on week 16, but showed that the mixture (ALVAC-HIV+gp160) (group #3) induced significantly higher neutralizing titers than the prime/boost
30 immunization (group #4) on week 26.

General conclusion

The present assay showed that the mixture vCP205 ($10^{6.5}$ CCID₅₀) plus gp160 (100 µg) elicited significantly higher gp160 and V3-specific responses than vCP205 or gp160 alone, and in some cases than the prime/boost immunization (vCP205/gp160 in alum), mainly after the final gp160 booster injection. The vCP205+gp16 mixture proved to be similarly immunogenic to gp160 adjuvanted in alum; given the low number of animals studied in the other groups. Moreover, the mixture appeared to evoke the best seroneutralizing responses to HIV-1-MN after the last fifth injection, although significance of this result could be proven only when compared with the prime/boost immunization, given the low number of animals in the other groups

Example 4: Immunogenicity of purified gp160 MN/LAI in the absence or presence of canarypox (ALVAC), in guinea-pigs

The experiment reported in the present Example 4 shows that both crude and purified non-recombinant ALVAC (CPpp) display adjuvant properties.

4A - CPpp preparations

CPpp (ALVAC) is derived from a canarypox strain isolated from a pox lesion on a infected canary, as described in Tartaglia et al, Virology (1992) 188: 217. CPpp is produced on chick embryo fibroblasts in DMEM-Ham F12, washed without serum and resuspended in lactoglutamate (crude CPpp). Instead of being resuspended in lactoglutamate, purified CPpp is obtained according to the purification process described in Joklick et al, Virology (1962) 18: 9.

4B- gp160 MN/LAI preparation

30

gp 160 preparations were achieved as described in example 1B

4C - Experimental procedure

Guinea pigs were submitted to immunization protocols as described in Table 4 hereinafter.

5

Table 4

Group (Guinea-pig #)	Primo-immunization (D1)			Booster (D29)
	gp160 dose (μ g)	ALVAC (CPpp)	ALVAC dose (CCID50)	gp160 dose (μ g)
1 (1,2,3,4,5)	5	None	0	5
2 (6,7,8,9,10)	5	Crude	10^6	5
3 (11,12,13,14,15)	5		10^6	5
4 (16,17,18,19,20)	5	Purified	10^7	5
5 (21,22,23,24,25)	5		10^8	5

Animals received both the primo and booster 1.10 ml doses intramuscularly (0.55 ml in each thigh) one month apart.

10

Serological analyses were carried out as described in Example 1C, using blood samples collected at days -1, 28 and 56.

15 4D- Serological analyses

Serological analyses were carried out with blood samples collected on days -1 (one day before the first immunization), 28, and 56. Antibodies to HIV gp160 glycoprotein and CPpp were titrated by ELISA using the same procedure as described in example 1C

20

4E - Serological results

Anti-CPpp antibodies (Figure 10a)

- 5 Four weeks after the first immunization, all the animals seroconverted (except group #1 which did not received any CPpp), and the titers remained stable after the gp160 booster till week 8.

Response to canarypox induced by 10^6 CCID₅₀ of crude CPpp was significantly higher
10 (+0.7 to 0.8 logs) than the one raised with the same dose of purified virus, was comparable to that elicited by 10^7 CCID₅₀ of purified CPpp, and was lower (~ -0.8 log) than that obtained with the dose of 10^8 CCID₅₀ of purified CPpp.

Anti-HIV gp160 MN/LAI antibodies (Figure 10b)

15

Anti-gp160 MN/LAI antibodies were elicited during the four weeks following the first injection in all animals, except some in group #5. In this group, which received a mixture of gp160 and 10^8 CCID₅₀ of purified CPpp, only 3 animals out of 5 seroconverted to gp160. For each guinea pig, a booster effect was noticeable after the second injection of
20 5 μ g of gp160.

The best anti-gp160 antibody responses were obtained in group #3, primed with gp160 mixed with the lowest dose (10^6 CCID₅₀) of purified CPpp. Indeed, this group displayed a significant increase in antibody titers (+0.8 and +0.9 logs at weeks 4 and 8,
25 respectively), comparatively to group #1 inoculated with the protein alone.

Co-injection of 10^7 CCID₅₀ of purified CPpp with gp160 (group #4) also enhanced the humoral response as compared to injection of the protein alone, but only on week 8 after the gp160 boost (+0.7 log). Surprisingly, in group #5 (gp160 mixed with 10^8 CCID₅₀ of
30 purified CPpp), a significant decrease in responding animals was observed (3 out of 5, versus 5 out of 5 in all other tested conditions). Moreover, the mean antibody titer (2.352 log) of the positive guinea pigs from group #5 was the lowest obtained in this assay.

Nevertheless, such a CPpp-induced inhibitory effect did not have any influence on the secondary response to gp160, which reached similar levels to those obtained in group #1.

Noticeably, addition of 10^6 CCID₅₀ of crude CPpp to gp160 did not improve the
5 antibody response as compared to gp160 alone.

General conclusion

This study clearly demonstrates an adjuvant effect of crude and purified CPpp on the
10 immunogenicity of gp160 MN/LAI inoculated IM in guinea pigs. Such a stimulation of the anti-gp160 antibody response was mostly observed at 10^6 CCID₅₀ of purified CPpp, whereas a marked inhibitory effect was noted at the higher dose of 10^8 CCID₅₀.

The results obtained with crude CPpp at 10^6 CCID₅₀ indicates that this CPpp
15 preparation does not seem to be able to enhance the anti-gp160 humoral response when combined with the 5 µg dose of the tested gp160. However, the same preparation does enhance the response to 1 µg gp160 (data not shown). Accordingly, the crude CPpp immunomodulating effect seems to be gp160-dose dependent.

20 Altogether, these findings show that both CPpp and gp160 must be used at optimal concentrations to see an adjuvant effect of canarypox. The present observation that both crude and purified CPpp can stimulate the anti-gp160 antibody response is in favor of the hypothesis that CPpp has intrinsic immuno-stimulating properties.

25 Example 5: Immunogenicity of gp160 MN/LAI in the presence of purified ALVAC-Luc (vCP292) inactivated or not, in guinea-pigs

5A - vCP297 preparation

30 vCP297 is an ALVAC vector derived from CPpp by homologous recombination so as to produce a vector in which the luciferase encoding sequence is placed under the control of an ALVAC promoter. vCP297 is produced and purified as described in Example 4A.

One ml of a vCP297 preparation exhibiting a mean titer of $10^{9.3}$ CCID50 on QT35 cells, was diluted 1/10 in PBS without Ca^{++} and Mg^{++} and inactivated at 56°C , 7 hours. It was then centrifuged during 5 hours at 10.000 rpm (centrifuge Sigman 201M) and the pellet and supernatant were harvested separately. The protein quantity and residual viral titer were quantified, being respectively 55 $\mu\text{g}/\text{ml}$ and $10^{3.5}$ CCID50/ml for the pellet and ≈ 1 $\mu\text{g}/\text{ml}$ and $10^{0.3}$ CCID50/ml for the supernatant.

5B - gp160 preparations were achieved as described in Example 1B.

10 5C - Experimental procedure

Guinea pigs were submitted to immunization protocols as described in Table 5 hereinafter.

15 Table 5

Group (Guinea-pig #)	First immunization (D1)		Booster (D29)
	gp160 MN/LAI doses (μg)	Purified ALVAC-Luc (vCP297)	gp160 MN/LAI doses (μg)
		Proteins (μg) Infectious dose (CCID50)	
1 (1, 2, 3, 4, 5)	5	0 0	5
2 (6, 7, 8, 9, 10)		0.055 10^5	
3 (11, 12, 13, 14, 15)		0.55 10^6	
4 (16, 17, 18, 19, 20)		pelleted fraction of the inactivated virus 0.55 $10^{1.5}$	
5 (21, 22, 23, 24, 25)		supernatant of the inactivated virus after centrifugation ¶ ~ 1 $= 10^{0.3}$	

Animals received the primo and booster doses under a final volume 1.10 ml, intramuscularly (0.55 ml in each thigh), one month apart.

- 5 Serological analyses were carried out as described in Example 1C, using blood samples collected at days -1, 28 and 56.

The isotypic distribution of the anti-gp160 humoral response was measured at day 56, using the procedure and conditions described in Examples 1C and 2C. The only
10 modification was the use of distinct peroxylase-conjugated goat antibodies specific for guinea-pig isotype IgG1 (Nordic, ref.: GAGp/IgG1/PO) or IgG2 (Nordic, ref.: GAGp/IgG2/PO), diluted 1/3.000 in PBS-Tween-milk.

5D - Serological results

15

Anti-CPpp antibodies (Figure 11a)

As previously observed, the humoral response induced against CPpp was dose-dependent: only 3 out of 5 guinea-pigs immunized with 10^5 CCID₅₀ of purified
20 ALVAC-Luc (vCP297) (group #2) weakly seroconverted to CPpp, whereas all animals (5 out of 5) that received 10^6 CCID₅₀ of the purified virus (group #3) developed a CPpp-specific response, and at much higher levels (mean ELISA titer in group #3 ~ 2 logs higher than in group #2).

- 25 The anti-CPpp titers elicited by the pelleted fraction of the inactivated ALVAC-Luc (group #4) were similar to those induced by the non-inactivated virus at equivalent protein quantity (group #3).

Surprisingly, the supernatant of inactivation of vCP297 (group #5) was also able to
30 mount an antibody response to the canarypox, and the titers induced were the highest observed in this assay. In particular, such a response differed in average by +0.6 and +0.9 log, on week 4 and 8 respectively, with that elicited by the non-inactivated purified virus (group #3). The high protein content present in this supernatant - measured subsequently

to inoculation - reaching $\sim 1 \mu\text{g}$ versus $0.55 \mu\text{g}$ for both the non-inactivated virus (group #3) or the pelleted fraction of the inactivated virus (group #4) could account for such results.

5 *Anti-gp160MN/LAI antibodies (Figure 11b)*

Anti-gp160MN/LAI antibodies were elicited in all animals during the four weeks following the first injection. For each guinea pig, an anamnestic response was noticeable after the gp160 booster injection.

10

While no significant difference in anti-gp160 antibody titers was detected between the five groups of guinea pigs after the primo-immunization, an enhancement of the humoral response to the HIV antigen was observed in some groups after the second inoculation. Indeed, by variance analysis using the Dunnett's t-test, the gp160-specific ELISA titers were found to be significantly higher in groups #3 and #4 than in group #1 (mean titers on week 8 in both groups #3 and 4 raised by $+0.7$ log as compared to group #1). In other words, these findings indicated that purified ALVAC-Luc, either inactivated or not, at protein quantity corresponding to 10^6 CCID₅₀ of infectious virus, had a significant adjuvant effect on the anti-gp160 antibody secondary response.

20

Priming with gp160 and purified ALVAC-Luc at 10^5 CCID₅₀ (group #2) also increased the anti-gp160 response (mean titers on week 8 raised by $+0.4$ log as compared to group #1), but such a stimulation was not found to be significant using the Dunnett's t-test.

25 By contrast, a significant adjuvant effect was detected in group #5, co-injected with gp160MN/LAI and the supernatant of inactivated purified ALVAC-Luc, (mean titers on week 8 raised by $+0.5$ log as compared to group #1), in accordance with the high protein content of ALVAC-Luc origin found in the supernatant.

30 Noticeably, the stimulating effect on the anti-gp160 humoral response associated to ALVAC-Luc, or products derived from it, was not found to be strictly related to the intensity of the anti-CPpp antibody response elicited. This confirms previous observations in Example 1, showing that high anti-CPpp titers were inversely related to

anti-gp160 antibody levels, probably as a consequence of antigenic competition between the HIV glycoprotein and the high doses of ALVAC injected.

IgG1 and IgG2 isotypic profiles of the anti-gp160 antibody response

5

The co-injection of gp160 and ALVAC-Luc, either inactivated or not (at protein quantity corresponding to 10^6 CCID₅₀ of infectious virus), was found to significantly increase the anti-gp160 antibody response of the IgG2 isotype, but not of the IgG1 one. Such an elevated IgG2 response was detected neither in group #2, that received gp160MN/LAI and 10^5 CCID₅₀ of purified recombinant canarypox, nor in group #5, injected with
10 gp160MN/LAI and the supernatant of inactivated ALVAC-Luc.

General conclusion

- 15 The data presented herein confirm those obtained in Example 1 with purified CPpp, showing that purified recombinant canarypox ALVAC-Luc (vCP297), when co-injected with gp160MN/LAI at the dose of 10^6 CCID₅₀ in guinea-pig, had also the capacity to significantly: (1) stimulate the gp160-specific IgG secondary response; and (2) influence the isotypic profile of the anti-gp160 antibodies (increase in specific IgG2 titers).
- 20 However, this adjuvant effect was detected earlier with CPpp than with vCP297 (*i.e.*, after the primo-immunization for the former *versus* only the gp160 boost for the latter), suggesting that recombinant ALVAC-Luc might be less effective in enhancing the humoral response than the parental vector.
- 25 Infectivity of ALVAC-Luc was not required for such a stimulating effect to occur, since both the non-inactivated and heat-inactivated recombinant canarypox, at equivalent protein quantity (corresponding to that contained in 10^6 CCID₅₀ of infectious virus), induced similar enhanced anti-gp160 antibody titers.
- 30 The observation that the supernatant of inactivated purified ALVAC-Luc also displayed an adjuvant effect on the anti-gp160 antibody response was unexpected, but could be explained by its high protein content of ALVAC-Luc origin. Its ability to elicit the

highest antibody titers against CPpp but not against gp160, confirms the results obtained in Example 1 using various doses of purified ALVAC.

Altogether, these findings are in line with the previous hypothesis that the
5 canarypoxvirus induces some immunomodulating effects *in vivo*.

Example 6: Immunogenicity and efficacy of a detergent-splitted monovalent A/Texas
flu vaccine in the absence or presence of canarypox (ALVAC) in mice

10 The experiment reported in the present example 6 shows that non-recombinant ALVAC increases the immunogenicity and the efficacy of a detergent-splitted flu vaccine essentially in aged immunocompromised mice.

6A- CPpp preparation

15

CPpp preparations were achieved as described in Example 4A. The titer of the stock CPpp preparation is $1.6 \cdot 10^9$ CCID 50/ml

6B- Detergent-splitted monovalent A/Texas flu vaccine preparation

20

The detergent-splitted monovalent A/Texas flu vaccine (A/Texas) was manufactured by Connaught laboratories and dialyzed against PBS before use, to eliminate residual detergent and formol from the vaccine.

25 6C- Serological analyses

Serological analyses were carried out with blood samples collected on days -4 (4 days before the first immunization), 14 and 35. Antibodies to HA were titrated as follows:
Wells of Maxisorp F96 NUNC plates were coated with 1 µg/ml of HA in a Carbonate
30 buffer 0.1M, pH 9.6 overnight at room temperature. Plates were then blocked for 1 hour at room temperature With 200 µl of 0.1% BSA (Bovine Serum Albumin) in PBS
Followed by 4 washings in washing buffer (PBS/ 0.1% Tween 20). All next incubations were carried out in a final volume of 100µl, followed by 5 washings in washing buffer.

Serial threefold dilutions of sera in dilution buffer (PBS/ 0.1% Tween 20/ 0.1% BSA) ranging from 1/100 to 1/218700 were added to the wells and incubated 60 min at 37 °C. After washings, a Sheep anti-mouse IgG1 peroxydase conjugated (Serotec) 1/15000 diluted or a Goat anti-mouse IgG2a Horseradish peroxydase conjugated (Caltag laboratories) 1/30000 diluted were added to the plates and incubated for another 60 min at 37°C. The plates were further washed and incubated for 20 min with O-phenylenediamine dihydrochloride (Sigma) at 1.5mg/ml in 0.05M phosphate citrate buffer, pH 5.0 containing 0.03% sodium perborate (Sigma). The colored reactions were stopped with 50µl of 4N H₂SO₄. Absorbance was read in a Titer Multiscan plate reader at 450 nm. The antibody titers were measured as the reciprocal of the last dilution at which the absorbance was 2 fold over the background absorbance obtained with pre-immune sera.

6D- Challenge

Randomized groups of mice were challenged on day 42 with 50µl of live mouse-adapted A/Taiwan/1/86 influenza virus (H1 strain) corresponding to 5 lethal doses 50 of virus (5 LD₅₀). The infectious doses were given intranasally after slight anesthesia of mice with Isoflurane. The protective immune responses induced by the tested vaccinal compositions were assessed by means of survival yields and weight changes that is a good parameter of morbidity. Mortality and weight changes in the mice were monitored daily and every pair day respectively up to 21 days after challenge. The article Suryaprakash S and al, (1997), 96: 157-169 is cited by reference for achieving experimental challenges.

6E- Immunization

Six randomized groups of 16-to-18 month old (aged) or 2-month old (young) DBA/2 mice were each submitted to one of the immunization protocol as described in Table 6 hereinafter. Each group is constituted with 6 mice.

Table 6

Group	Primo-immunization		Boost	
	A/Texas dose (in μg)	ALVAC (CPpp) dose (in CCID 50)	A/Texas dose (in μg)	ALVAC (CPpp) dose (in CCID 50)
1	3	0	3	0
2	3	2×10^7	3	2×10^7
3	0	2×10^7	0	2×10^7

- 5 The groups were primed and boosted, via the S.C. route, with the compositions in a final volume of 0.2 ml. For immunization of group 2, A/Texas and appropriate amount of ALVAC were mixed together with appropriate amount of PBS to bring the final injected volume to 0.2 ml per mouse. The booster immunization was carried out in all groups one month later.

10

6F- Serological results

Anti A/Texas IgG1 antibodies (Figure 12)

- 15 Anti-A/Texas IgG1 antibodies were elicited during the two weeks following the first injection in 3 to 6 mice from group 1, in 5 to 6 mice from group 2, whereas no specific IgG1 were elicited in mice primed with ALVAC alone (group 3). The specific IgG1 mean titer was approximately 10 fold higher in the group of mice primed with the mixture of A/Texas and CPpp (group 1) than that observed in the group of mice given
- 20 A/Texas alone (group 2). The boost did not change the distribution pattern of specific IgG1 responses (observed in the 3 groups of mice) during the 15 days following the second injection. However, the specific IgG1 mean titers of groups 1 and 2 were ten-fold higher.

Anti A/Texas IgG2a antibodies (Figure 13)

Anti-A/Texas IgG2a antibodies were elicited during the two weeks following the first
5 injection in 3 to 6 mice from group 1, in 5 to 6 mice from group 2, whereas no specific
IgG2a were elicited in mice primed with ALVAC alone (group 3). The specific IgG2a
mean titer was approximately 10 fold higher in the group of mice primed with the
mixture of A/Texas and CPpp (group 1) than that observed in the group of mice given
A/Texas alone (group 2). The boost did not change the distribution pattern of specific
10 IgG2a responses (observed in the 3 groups of mice) during the 15 days following the
second injection. However, the specific IgG2a mean titers of groups 1 and 2 were ten-
fold higher.

General conclusion

15

This study clearly demonstrates an adjuvant effect of CPpp on the immunogenicity of
A/Texas inoculated subcutaneously in immunocompromised aged mice. A similar
enhancer supportive effect of CPpp on the immunogenicity of A/Texas is also observed
in young mice. It is also worth noticing that CPpp increases both specific IgG2a and
20 IgG1 responses in old mice immunized with the mixture of ALVAC and A/Texas; which
means that CPpp could act both on TH1 (T helper 1) and TH2 (T helper 2) immune
responses. Indeed, it is well understood for a man skilled in the art that the TH2 immune
response correlates with the level of specific IgG1 response in mice and is featured by a
rather humoral immune response, whereas the TH1 immune response correlates with the
25 level of specific IgG2a response and is commonly featured by a cytotoxic and
inflammatory immune response. In conclusion, this reveals that CPpp acts both on
specific cellular and humoral immune responses, when it is concomitantly administered
with an immunogenic compound.

30

6G- Challenge results (figures 14 and 15)

Mortality (Figure14)

5 Three weeks after the boost, all the aged mice were given intranasally a lethal challenge of live influenza virus. All the 6 mice of the group 3 (group receiving CPpp alone) died during the 8 days consecutive to challenge. Only, 1 of 6 mice (16% survival rate) of the group 1 (group receiving A/Texas alone) was still alive 20 days after challenge whereas 4 of 6 mice of the group 2 (group receiving the mixture A/Texas and CPpp) (66% survival rate) were still alive. Moreover, the survival curve of group 2 clearly shows that the two deaths observed were delayed compared to those observed in groups 1 and 3 (Figure 14)

Morbidity (Figure15)

15 The morbidity of mice after challenge was monitored for 20 days and assessed by the weight loss rate. The weight loss occurred shortly after the challenge in the group of mice immunized with CPpp alone (group 3) reaching up to 35% of the initial weight. Mice immunized with A/Texas alone (group 1) also showed a severe weight loss after challenge similar to that observed in group 3. The weight loss rate curve during the 20 days of the monitoring for the only one survivor of group 1 is represented in Figure 15 and clearly shows that the weight loss was fast and severe, whereas the weight recovery was much slower. On the other hand, the weight loss rate curve involving the 4 survivors of group immunized with the mixture of ALVAC and A/Texas (group 2) shows improvements over group 1. First, the maximum weight loss rate did not exceed 15% of the initial weight and second, the weight recovery was faster, since the survivors had recovered their initial weight by the end of the monitoring.

Although morbidity and mortality results about aged mice only are reported here, it is indicated that similar results were obtained with young mice.

30 **General Conclusion**

Morbidity and mortality results obtained with the live influenza challenge model are in agreement with those obtained from immunogenicity studies and show that ALVAC is

not only able to enhance the specific immune response to A/Texas but also contributes to the elicitation of a specific protective immune response, when it is co-administered with an antigen from a pathogenic micro-organism.

Claims

1. The use of a poxvirus in the manufacture of a pharmaceutical composition comprising an immunogenic compound for inducing an immune response in a vertebrate, wherein the poxvirus is able to enhance a specific immune response to the immunogenic compound.
5
2. The use according to claim 1, of a poxvirus in the manufacture of a pharmaceutical composition comprising an immunogenic compound, which comprises at least one antigenic determinant characteristic of a pathogenic microorganism or a tumor-associated antigen.
10
3. The use according to claim 2, of a poxvirus in the manufacture of a pharmaceutical composition comprising an immunogenic compound, which comprises at least one antigenic determinant characteristic of a pathogenic microorganism or a tumor-associated antigen; wherein the pharmaceutical composition induces a protective immune response against the pathogenic microorganism or the tumor.
15
4. The use according to claim 3, wherein the pharmaceutical composition is intended to treat or prevent an infectious disease induced by the pathogenic microorganism or a tumor-associated disorder.
20
5. The use according to any one of claims 1 to 4, of a poxvirus in the manufacture of a pharmaceutical composition comprising an immunogenic compound which is a peptide or a polypeptide.
25
6. The use according to claim 5, of a poxvirus in the manufacture of a pharmaceutical composition comprising an immunogenic compound, which is an HIV or influenza virus peptide or polypeptide.
30
7. The use according to claim 1, of a poxvirus in the manufacture of a pharmaceutical composition comprising an immunogenic compound, which is a recombinant DNA plasmid encoding a peptide or a polypeptide which comprises at least one antigenic

determinant characteristic of a pathogenic microorganism or a tumor-associated antigen.

- 5 8. The use according to claim 7, wherein the pharmaceutical composition induces a protective immune response against the pathogenic microorganism or the tumor.
9. The use according to claim 8, wherein the pharmaceutical composition is intended to treat or prevent an infectious disease induced by the pathogenic microorganism or a tumor-associated disorder.
- 10 10. The use according to any one of claims 7 to 9, of a poxvirus in the manufacture of a pharmaceutical composition comprising an immunogenic compound, which is a recombinant DNA plasmid encoding an HIV or Influenza virus peptide or polypeptide.
- 15 11. The use according to claim 1, of a poxvirus in the manufacture of a pharmaceutical composition comprising an immunogenic compound, which is an hapten coupled to a carrier molecule
- 20 12. The use according to any one of claims 1 to 11, wherein the poxvirus is a live virus.
13. The use according to claim 12, wherein the poxvirus is an attenuated virus.
- 25 14. The use according to any one of claims 1 to 11, wherein the poxvirus is an inactivated virus.
15. The use according to any one of claims 1 to 14, wherein the poxvirus does not encode any heterologous polypeptide.
- 30 16. The use according to any one of claims 1 to 13, wherein the poxvirus encodes an heterologous polypeptide.

17. The use according to claim 16, wherein the poxvirus encodes an heterologous polypeptide, which is selected from the group consisting of adhesion molecules, co-immunostimulatory molecules, apoptotic factors, cytokines and growth hormones.
- 5
18. The use according to claims 16 and 5 or 6, wherein the pharmaceutical composition comprises an immunogenic compound, which is a peptide or a polypeptide and, wherein the poxvirus encodes an heterologous polypeptide, which is immunologically cross-reactive with the immunogenic compound.
- 10
19. The use according to claim 18, wherein the poxvirus encodes an heterologous polypeptide, which has an amino acid sequence identical to the amino acid sequence of the immunogenic compound.
- 15
20. The use according to any one of claims 1 to 19, wherein the poxvirus is selected from the group consisting of orthopoxvirus, avipoxvirus, capripoxvirus, suipoxvirus, molluscipoxvirus, yatapoxvirus or an entomopoxvirus.
21. The use according to claim 20, wherein the poxvirus is a vaccinia virus.
- 20
22. The use according to claim 20, wherein the poxvirus is a canarypox virus.
23. A pharmaceutical composition comprising (i) an immunogenic compound and (ii) a poxvirus encoding an heterologous polypeptide which is selected from the group consisting of adhesion molecules, co-immunostimulatory molecules, chemokines apoptotic factors, cytokines and growth hormones.
- 25
24. A pharmaceutical composition according to claim 23, wherein the immunogenic compound is selected from the group consisted of a peptide, a polypeptide, a DNA plasmid encoding a peptide or a polypeptide, and an hapten coupled to a carrier molecule.
- 30

25. A pharmaceutical composition comprising (i) an immunogenic compound which is a first polypeptide, and (ii) a poxvirus encoding a second heterologous polypeptide, which has an amino acid sequence identical to the amino acid sequence of the first polypeptide.
- 5
26. A pharmaceutical composition comprising (i) an immunogenic compound which is a DNA plasmid encoding a first polypeptide, and (ii) a poxvirus encoding a second heterologous polypeptide, which has an amino acid sequence identical to the amino acid sequence of the first polypeptide.
- 10
27. A pharmaceutical composition according to claim 24, 25 or 26, wherein the first and second polypeptide polypeptide are HIV or influenza virus polypeptides.
- 15
28. A pharmaceutical composition according to any one of claims 23 to 27, further comprising a pharmaceutical acceptable diluent or carrier.

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Figure 1a

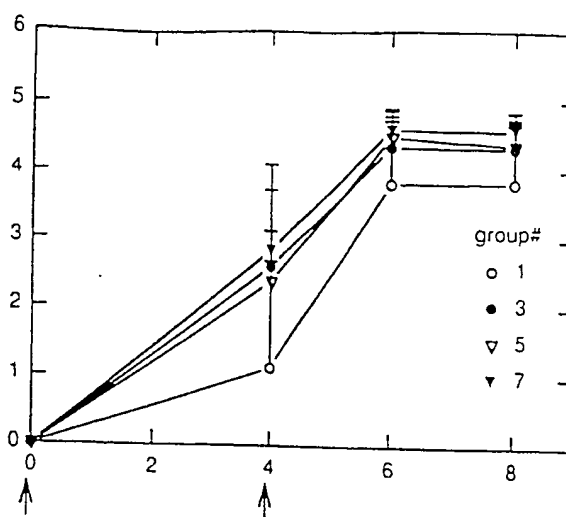
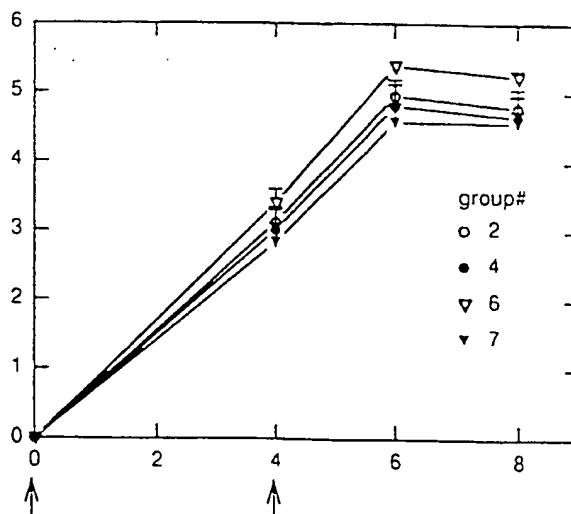


Figure 1b



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Figure 2a

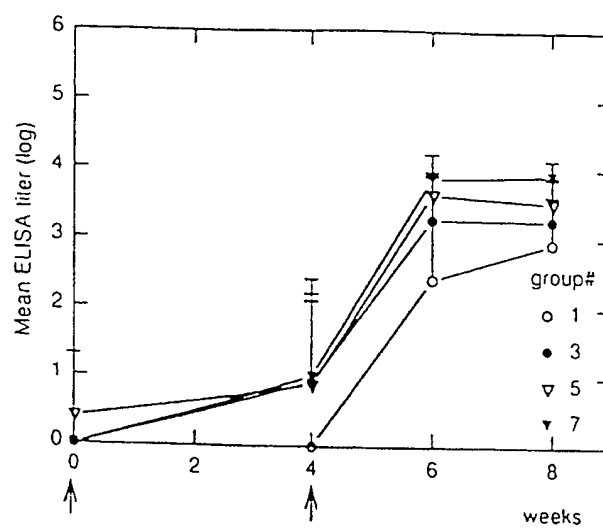
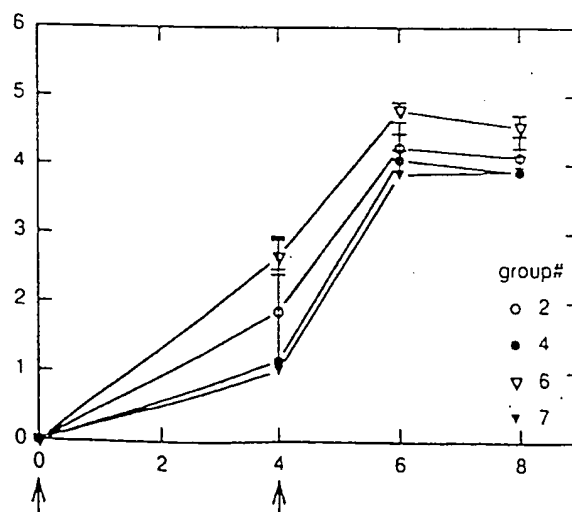
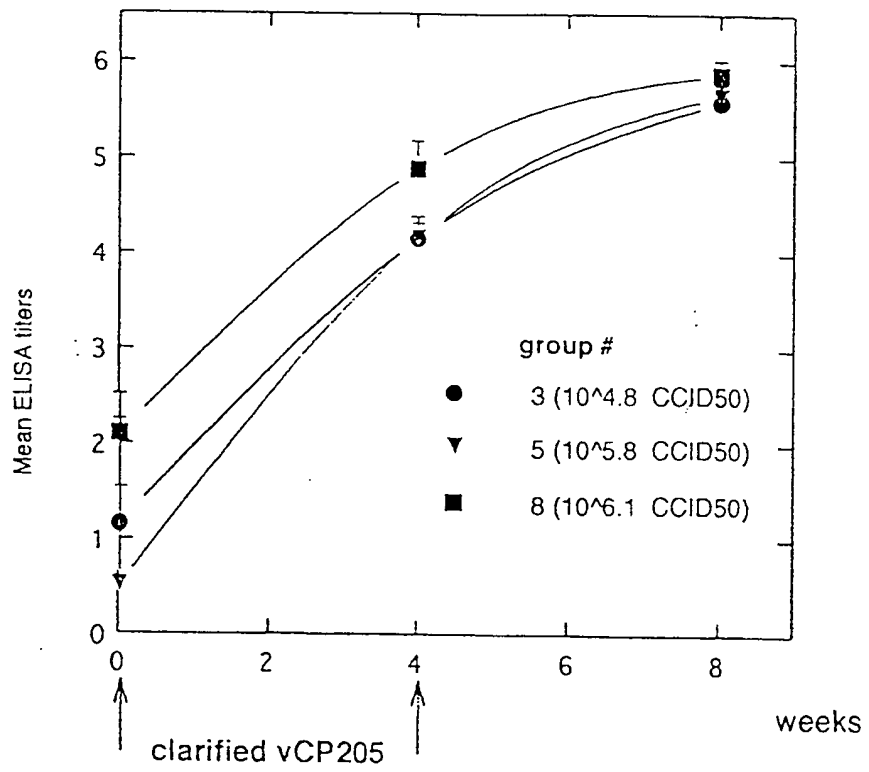


Figure 2b



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Figure 3



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Figure 4a

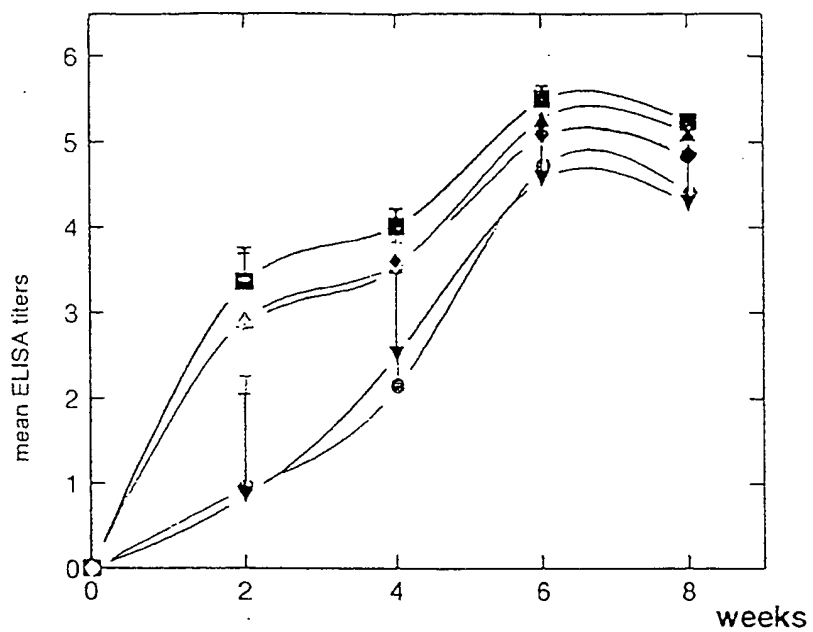
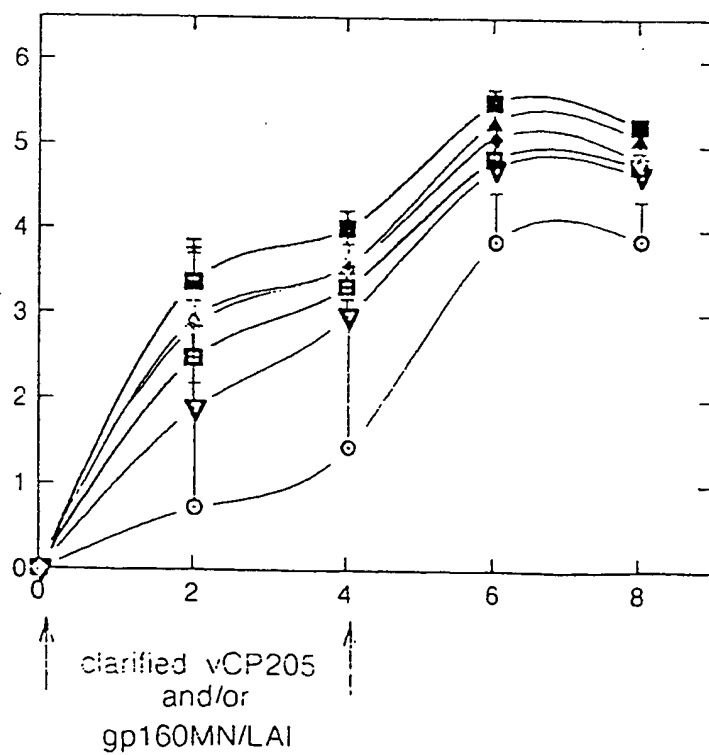


Figure 4b



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Figure 5

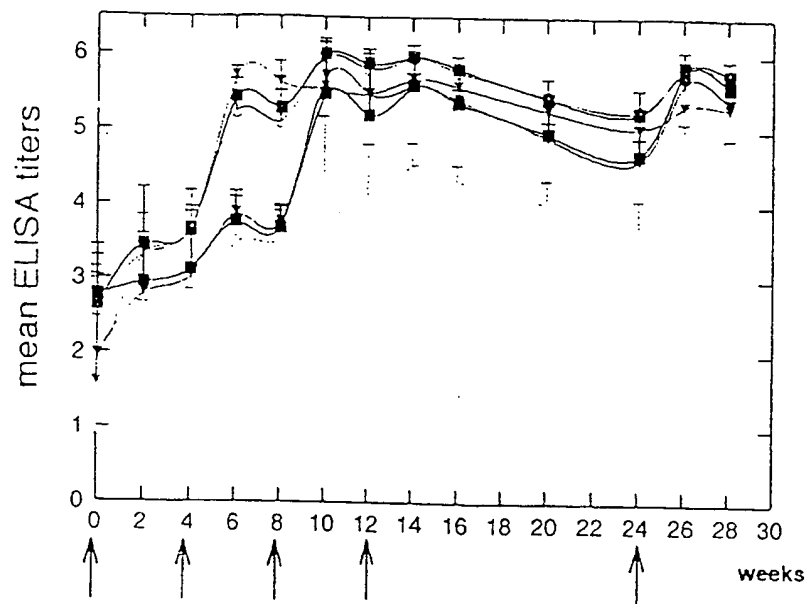
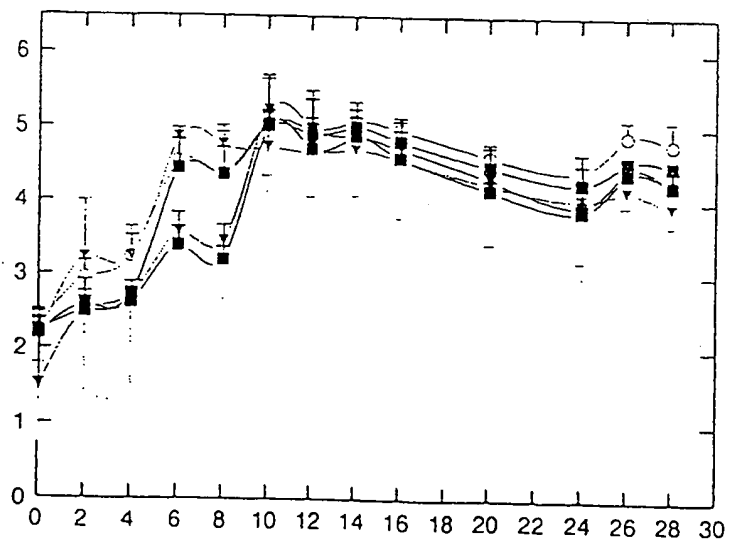


Figure 6



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Figure 7

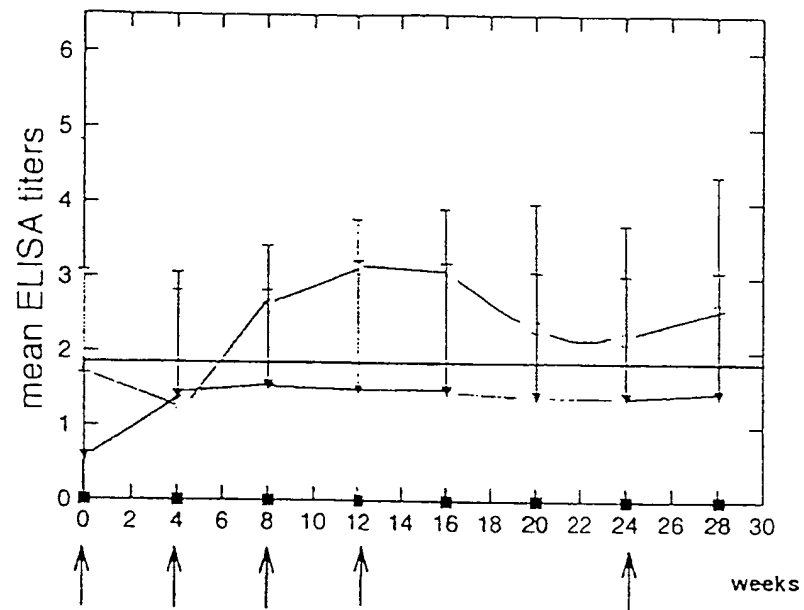
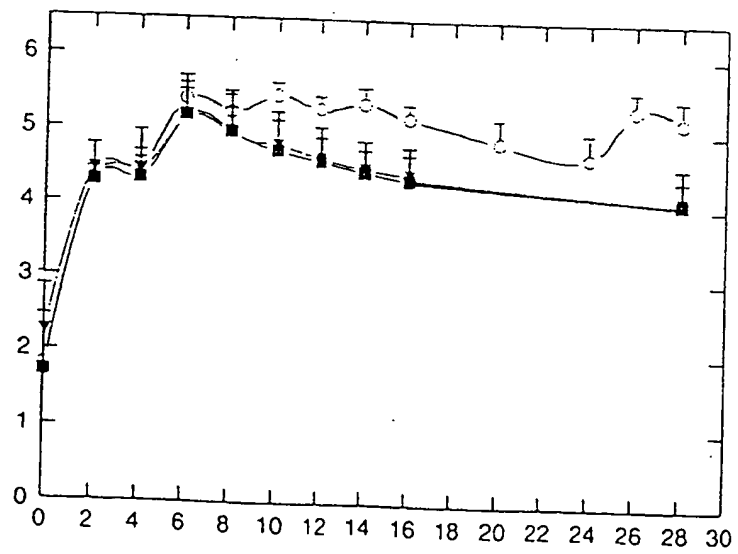
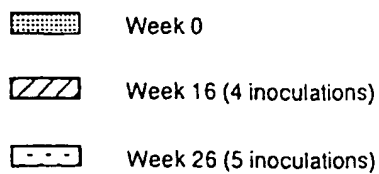
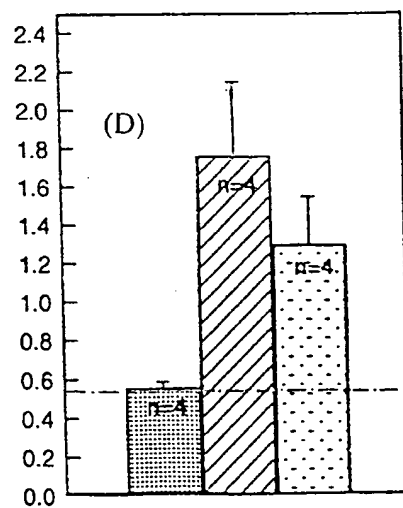
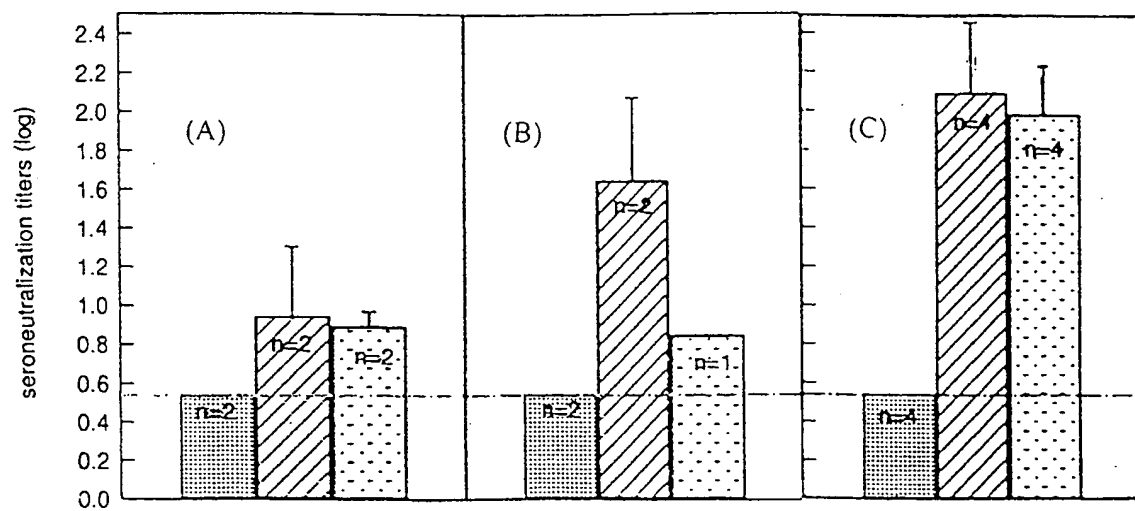


Figure 8



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Figure 9



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Figure 10a

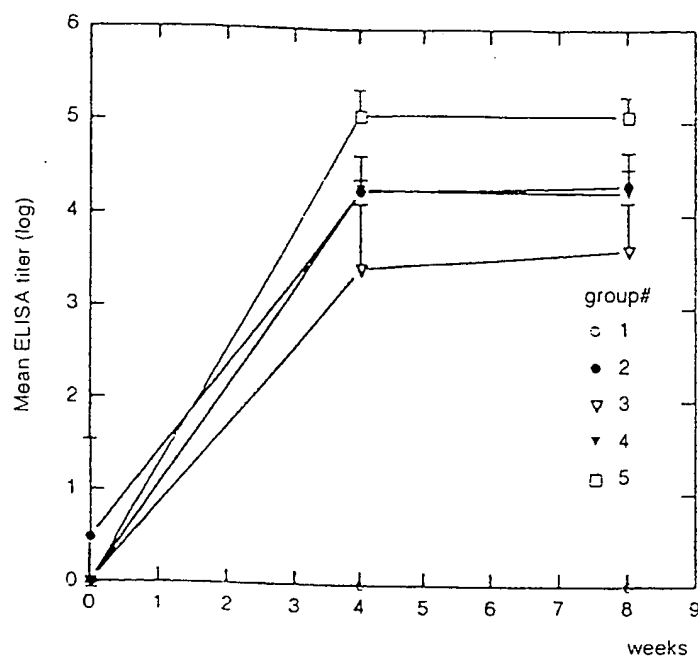
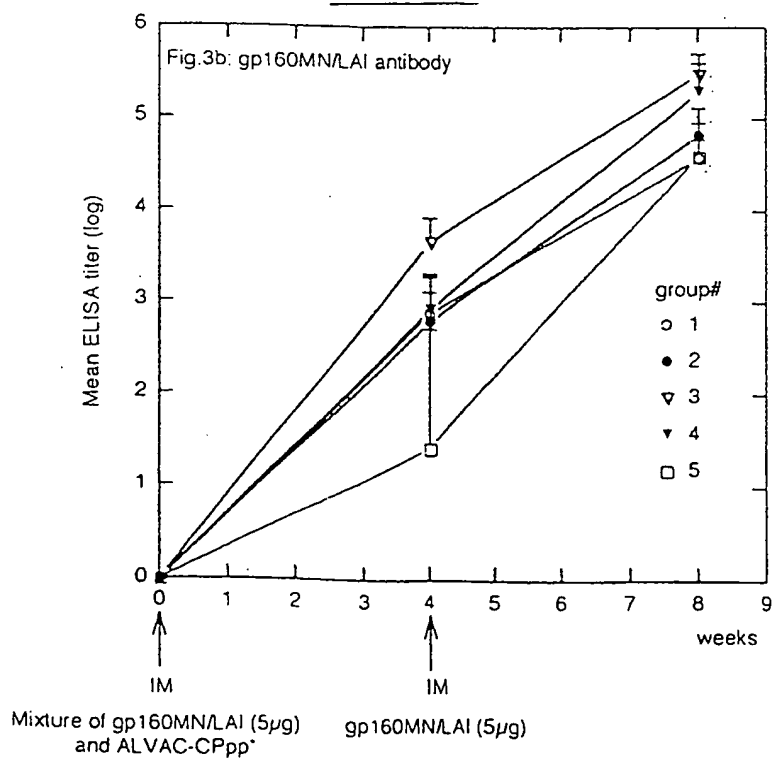
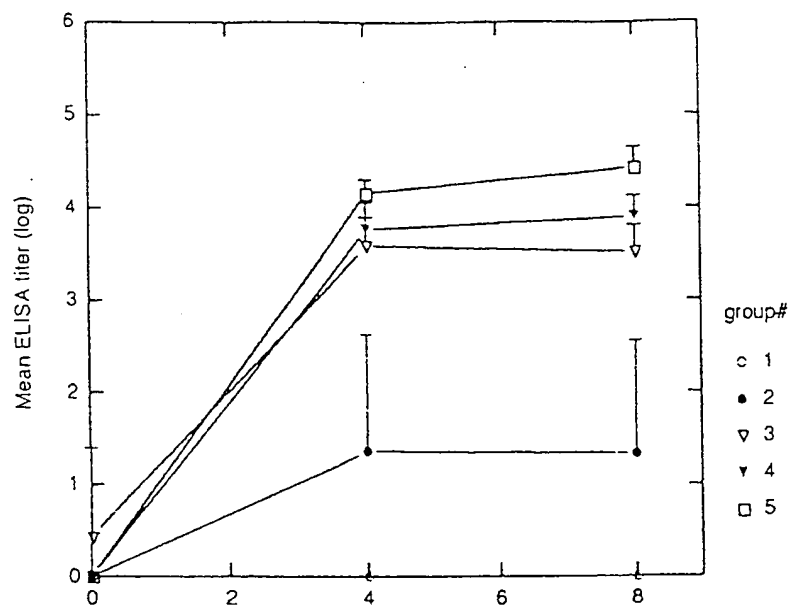
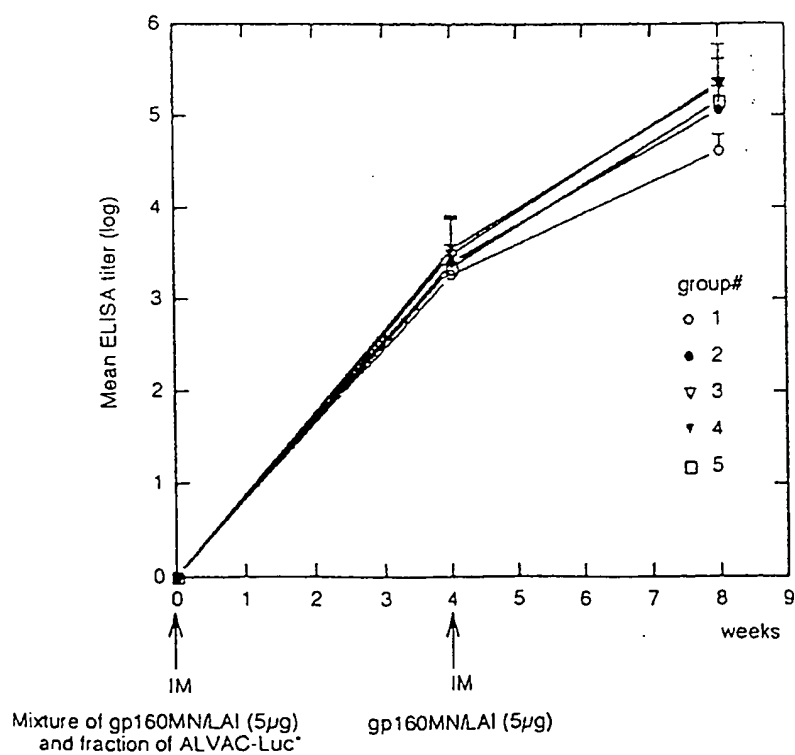


Figure 10b

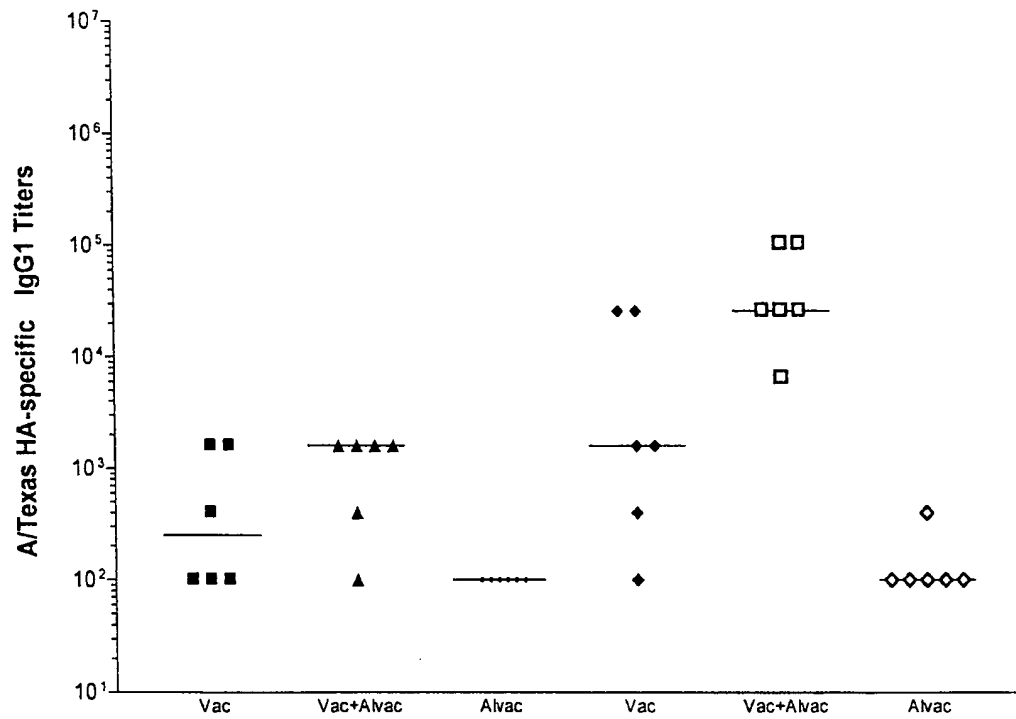


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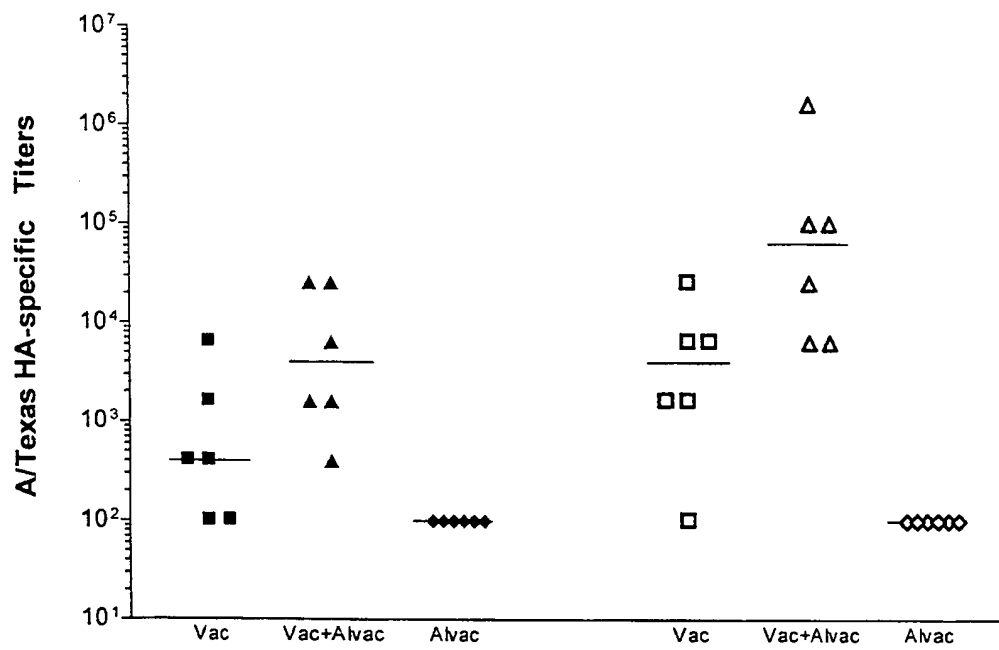
Figure 11aFigure 11b

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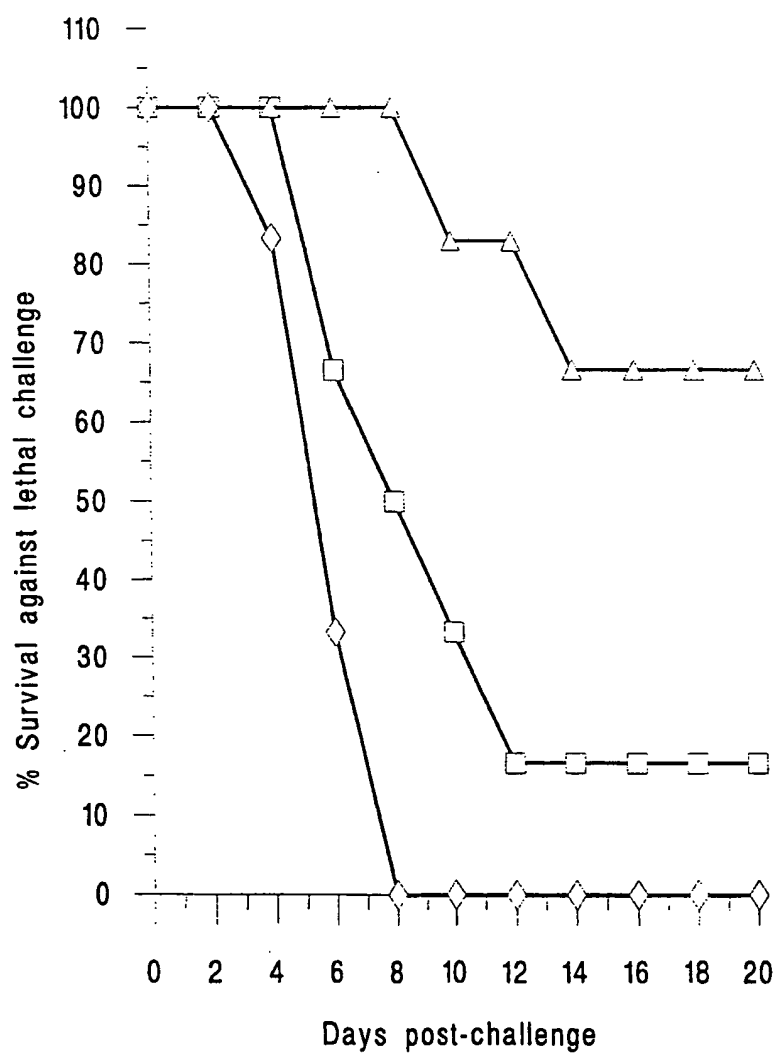
Figure12



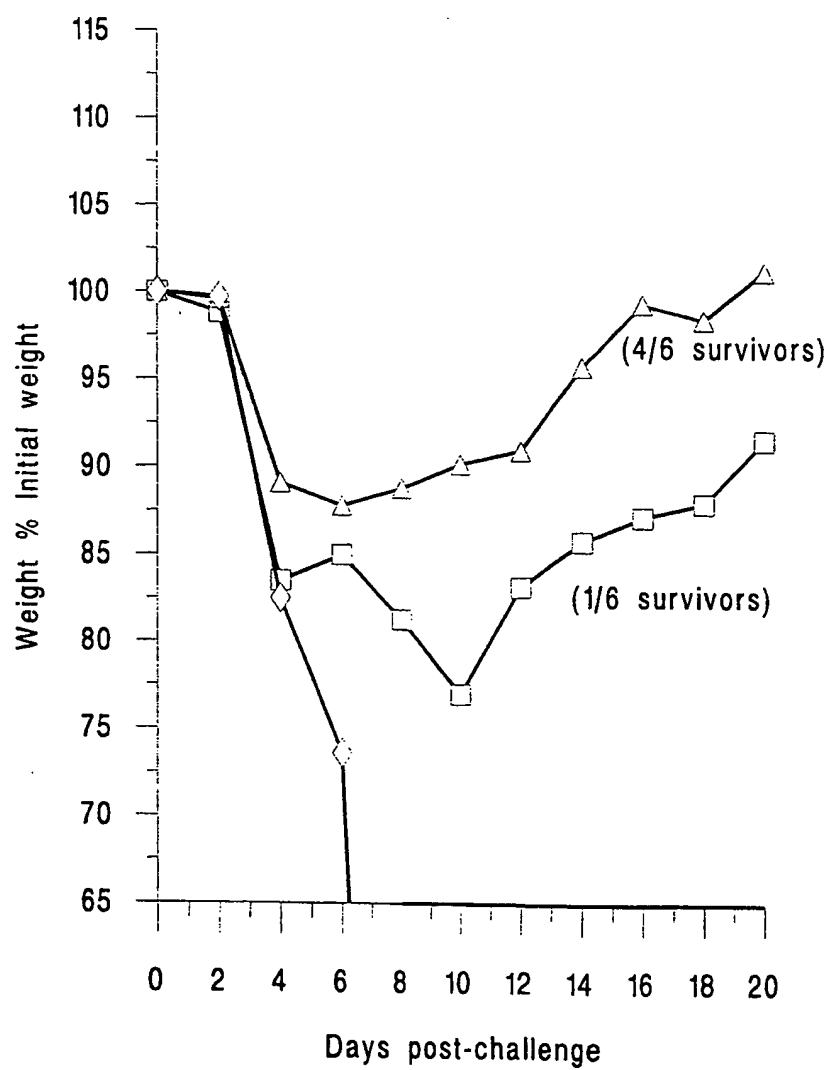
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Figure 13

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Figure 14

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Figure 15

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 39/39, 39/21, 35/76, 39/145, 39/00	A3	(11) International Publication Number: WO 00/00216 (43) International Publication Date: 6 January 2000 (06.01.00)
(21) International Application Number: PCT/EP99/04913 (22) International Filing Date: 28 June 1999 (28.06.99) (30) Priority Data: 98420110.3 26 June 1998 (26.06.98) EP 98420111.1 26 June 1998 (26.06.98) EP (71) Applicant (for all designated States except US): PASTEUR MERIEUX SERUMS ET VACCINS [FR/FR]; 58, avenue Leclerc, F-69007 Lyon (FR). (72) Inventors; and (75) Inventors/Applicants (for US only): CHEVALIER, Michel [FR/FR]; 19, rue de la Guillotière, F-38270 Beaurepaire (FR). MEIGNIER, Bernard [FR/FR]; 26, rue du 8 mai 45, F-69510 Thurins (FR). MOSTE, Catherine [FR/FR]; 7, avenue Louis Momet, F-69260 Charbonnières-les-Bains (FR). SAMBHARA, Suryaprakash [CA/CA]; 50 Harness Circle, Markham, Ontario L3S 1Y1 (CA). (74) Agent: AYROLES, Marie-Pauline; Pasteur Mérieux Sérums et Vaccins, 58, avenue Leclerc, F-69007 Lyon (FR).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 16 March 2000 (16.03.00)
(54) Title: USE OF POXVIRUSES AS ENHANCER OF SPECIFIC IMMUNITY		
(57) Abstract <p>The invention relates to a method for enhancing the specific immune response against an immunogenic compound which comprises administering the immunogenic compound together with a poxvirus recombinant and a vaccinal antigen, which is not a poxvirus. The immunological material may be any biological material useful as a vaccine <i>e.g.</i>, a polypeptide characteristic of a pathogenic microorganism or associated with a tumoral disorder, a DNA plasmid encoding a peptide or a polypeptide characteristic of a pathogenic microorganism or a tumor-associated antigen, or an hapten coupled to a carrier molecule. The poxvirus may be a live, attenuated or inactivated virus or a recombinant virus. Recombinant virus may encode a heterologous polypeptide such as chemokines, cytokines or co-immunostimulatory molecules or an homologous polypeptide, which is immunologically cross reactive with the immunogenic polypeptide or peptide.</p>		

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/EP 99/04913

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 A61K39/39 A61K39/21 A61K35/76 A61K39/145 A61K39/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 A61K C07K C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
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C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A. MAYR ET AL.: "BEKÄMPFUNG DES ECTHYMA CONTAGIOSUM (PUSTULARDERMATITIS) DER SCHAFE MIT EINEM NEUEN PARENTERAL-ZELLKULTUR-LEBENDIMPFSTOFF." ZENTRALBLATT FÜR VETERINÄRMEDIZIN, REIHE B, vol. 28, no. 7, 1981, pages 535-552, XP002086053 BERLIN, DE page 545, paragraph 4 -page 546, paragraph 2	1-4, 14, 15, 20
X	WO 95 22978 A (A. MAYR) 31 August 1995 (1995-08-31) page 15, line 24 -page 16, line 6; claims; figure 2; examples 6,10; table 3 page 20, line 1 - line 18 <div style="text-align: center;">-/--</div>	1-5, 12-16, 20-22
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
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Date of the actual completion of the international search <div style="text-align: center;">17 December 1999</div>		Date of mailing of the international search report <div style="text-align: center;">12/01/2000</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo.nl, Fax: (+31-70) 340-3016		Authorized officer <div style="text-align: center;">Ryckebosch, A</div>

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/04913

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 40268 A (AMERICAN HOME PRODUCTS CORPORATION) 19 December 1996 (1996-12-19) page 6, paragraph 4 -page 7, line 3; claims 1-5,12,13 ---	1-5,7-9, 12-15, 20-22
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Y	WO 93 08836 A (INSTITUT PASTEUR) 13 May 1993 (1993-05-13) page 3, line 4 - line 8; claims 1-4,8,28-30,39-42,46,49,50 page 13, line 12 - line 30 page 16, paragraph 4 -page 17, paragraph 3 ---	1-28
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International Application No

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(72) Inventors; and

(75) Inventors/Applicants (for US only): **CHEN, Ling** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). **SHIVER, John** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).

BETT, Andrew, J. [CA/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). **CASIMIRO, Danilo, Riguera** [PH/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). **CAULFIELD, Michael, J.** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). **CHASTAIN, Michael, A.** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). **EMINI, Emilio, A.** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).

(74) Common Representative: **MERCK & CO., INC.**; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).

(81) Designated States (national): CA, JP, US.

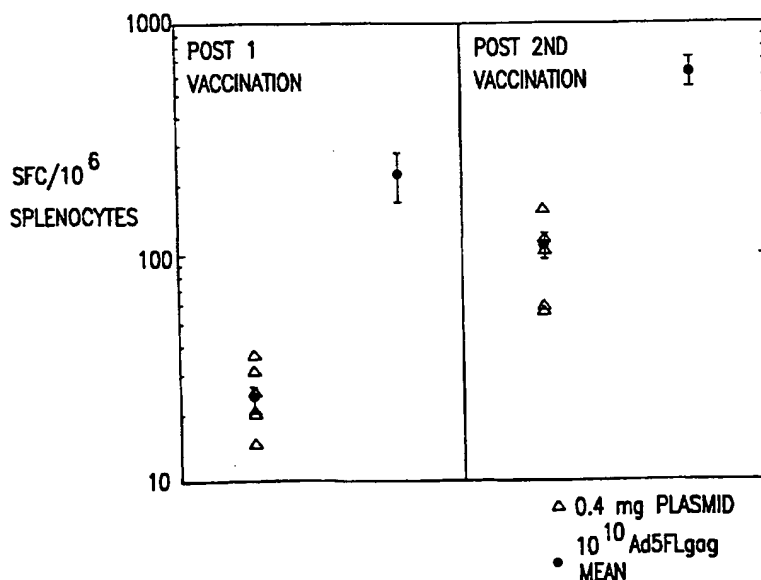
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ADENOVIRUS CARRYING GAG GENE HIV VACCINE



(57) Abstract: An adenoviral vector is described which carries a codon-optimized gag gene, along with a heterologous promoter and transcription terminator. This viral vaccine can effectively prevent HIV infection when administered to humans either alone or as part of a prime and boost regime also with a vaccine plasmid.

WO 01/02607 A1

ADENOVIRUS CARRYING GAG GENE HIV VACCINE

FIELD OF THE INVENTION

This invention relates to replication deficient adenovirus vectors
5 comprising an optimized human immunodeficiency virus (HIV) gag gene under the
control of a strong promoter, which are suitable for vaccines against HIV.

BACKGROUND OF THE INVENTION

Human Immunodeficiency Virus-1 (HIV-1) is the etiological agent of
10 acquired human immune deficiency syndrome (AIDS) and related disorders.

Vaccination is an effective form of disease prevention and has proven
successful against several types of viral infection. However, determining ways to
present HIV-1 antigens to the human immune system in order to evoke protective
humoral and cellular immunity is a difficult task. In AIDS patients, free virus is
15 present in low levels only. Transmission of HIV-1 is enhanced by cell-to-cell
interaction via fusion and syncytia formation. Hence, antibodies generated against
free virus or viral subunits are generally ineffective in eliminating virus-infected cells.

European Patent Applications 0 638 316 (Published February 15,
1995) and 0 586 076 (Published March 9, 1994), (both assigned to American Home
20 Products Corporation) describe replicating adenovirus vectors carrying an HIV gene,
including *env* or *gag*. Various treatment regimens were used with chimpanzees and
dogs, some of which included booster adenovirus or protein plus alum treatments.

Infection with HIV-1 is almost always fatal, and at present there are no
cures for HIV-1 infection. Effective vaccines for prevention of HIV-1 infection are
25 not yet available. Because of the danger of reversion or infection, live attenuated
virus probably cannot be used as a vaccine, and subunit vaccine approaches have not
been successful at preventing HIV infection. Treatments for HIV-1 infection, while
prolonging the lives of some infected persons, have serious side effects. There is thus
a great need for effective treatments and vaccines to combat this lethal infection.

30

SUMMARY OF THE INVENTION

This invention relates to a vaccine composition comprising a
replication- defective adenoviral vector comprising at least one gene encoding an HIV
gag protein, wherein the gene comprises codons optimized for expression in a human,
35 and the gene is operably linked to a heterologous promoter.

Another aspect of this invention relates to an adenoviral vaccine vector comprising: a replication defective adenoviral genome, wherein the adenoviral genome does not have a functional E1 gene, and the adenoviral genome further comprises a gene expression cassette comprising:

- 5 i) a nucleic acid encoding a HIV gag protein, wherein the nucleic acid is codon optimized for expression in a human host;
- ii) a heterologous promoter is operatively linked to the nucleic acid encoding the gag protein; and
- iii) a transcription terminator .

10 In preferred embodiments, the E1 gene has been deleted from the adenoviral vector, and the HIV expression cassette has replaced the deleted E1 gene. In other preferred embodiments, the replication defective adenovirus genome does not have a functional E3 gene, and preferably the E3 gene has been deleted.

This invention also relates to a shuttle plasmid vector comprising: an
15 adenoviral portion and a plasmid portion, wherein said adenovirus portion comprises:
a) a replication defective adenovirus genome which does not have a functional E1 gene; and b) a gene expression cassette comprising: a nucleic acid encoding an HIV gag protein, wherein the nucleic acid is codon optimized for expression in a human host; a heterologous promoter operably linked to the nucleic acid encoding the gag
20 protein; and a transcription terminator.

Other aspects of this invention include a host cell comprising the adenoviral vaccine vectors and/or the shuttle plasmid vectors, methods of producing the vectors comprising introducing the adenoviral vaccine vector into a host cell which expresses adenoviral E1 protein, and harvesting the resultant adenoviral
25 vaccine vectors.

Another aspect of this invention is a method of generating a cellular immune response against an HIV protein in an individual comprising administering to the individual an adenovirus vaccine vector comprising:

- a) a replication defective adenoviral vector, wherein the adenoviral
30 vector does not have a functional E1 gene, and
- b) a gene expression cassette comprising: i) a nucleic acid encoding an HIV gag protein, wherein the nucleic acid is codon optimized for expression in a human host; ii) a heterologous promoter operatively linked to the nucleic acid encoding the gag protein; and iii) a transcription terminator.

In some embodiments of this invention, the individual is given more than one administration of adenovirus vaccine vector, and it may be given in a regiment accompanied by the administration of a plasmid vaccine. The plasmid vaccine comprises a plasmid encoding a codon-optimized gag protein, a heterologous promoter operably linked to the gag protein nucleic acids, and a transcription terminator. There may be a predetermined minimum amount of time separating the administrations. The individual can be given a first dose of plasmid vaccine, and then a second dose of plasmid vaccine. Alternatively, the individual may be given a first dose of adenovirus vaccine vector, and then a second dose of adenovirus vaccine vector. In other embodiments, the plasmid vaccine is administered first, followed after a time by administration of the adenovirus vector vaccine. Conversely, the adenovirus vaccine vector may be administered first, followed by administration of plasmid vaccine after a time. In these embodiments, an individual may be given multiple doses of the same adenovirus serotype in either viral vector or plasmid form, or the virus may be of differing serotypes.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 is a graph showing the number of gag peptide-specific interferon-gamma secreting splenocytes ($\times 10^6$) from rats which were immunized with gag plasmid or Ad5FLgag.

FIGURE 2 shows serum SEAP (secreted alkaline phosphatase) expression levels in rhesus monkeys following injection with FG Ad5-SEAP or SEAP DNA constructs.

FIGURES 3A, 3B and 3C show anti-HIV gag cytotoxic T lymphocyte responses in three rhesus monkeys vaccinated with FG Ad5 tPAgag. Each panel represents the specific killing response of a particular monkey (denoted as numbers 92x024 in FIGURE 3A, 94x012 in FIGURE 3B, and 94x025 in FIGURE 3C) at various time points following immunization at 0, 8, and 24 weeks. The abscissa axis shows the effector/target (E/T) ratios of cultured T cells and B cells employed in this assay, while the ordinate axis shows specific lysis values obtained for each sample. Specific lysis values of at least 10% difference between curves \pm gag peptide antigen are generally considered significant. The square symbols represent target cells treated with an irrelevant influenza peptide antigen while the circles, triangles, and diamonds represent target cells treated with partial or complete gag peptide pools, respectively.

FIGURES 4A-H show anti-HIV gag cytotoxic T lymphocyte responses in rhesus monkeys vaccinated with FG Ad5FLgag. Figures 4A, B, and C are the first group of monkeys, D,E,and F are the second, and G, H, and I are the third group. Each represents specific killing responses of each monkey receiving the indicated treatment. The abscissa axis shows the effector/target (E/T) ratios of cultured T cells and B cells employed in this assay, while the ordinate axis shows specific lysis values obtained for each sample. Specific lysis values of at least 10% difference between curves \pm gag peptide antigen are generally considered significant. The square symbols represent target cells treated with DMSO alone at the same concentration as samples containing peptides while the circles, triangles, and diamonds represent target cells treated with partial (F, G) or complete (H) gag peptide pools, respectively.

FIGURES 5A-H show anti-HIV gag cytotoxic T lymphocyte responses in rhesus monkeys vaccinated with Ad2Flgag priming, followed by either Ad2Flgag or Ad5Flgag boosting. Each panel (Figures 5A-G) represents specific killing responses of a group of three monkeys receiving the indicated treatment. The last panel (Figure 5H) shows responses from two naive monkeys that were not vaccinated. The abscissa axis shows the effector/target (E/T) ratios of cultured T cells and B cells employed in this assay, while the ordinate axis shows specific lysis values obtained for each sample. Specific lysis values of at least 10% difference between curves \pm gag peptide antigen are generally considered significant..

FIGURE 6 is the nucleic acid sequence (SEQ.ID.NO.1) of the optimized human HIV-1 gag open reading frame.

FIGURE 7A shows construction of the adenovirus carrying codon-optimized gag. FIGURE 7B shows construction of the adenovirus carrying codon-optimized tPA-gag.

FIGURE 8 is the nucleic acid sequence of the optimized tPA-gag open reading frame.

As used throughout the specification and claims, the following definitions and abbreviations are used:

In general, adenoviral constructs, gene constructs are named by reference to the genes contained therein, such as below:

"tPAgag" refers to a fusion between the leader sequence of the tissue plasminogen activator leader sequence and an optimized HIV gag gene. .

"Ad5-tPAgag" refers to an adenovirus serotype 5 replication deficient virus which carries an expression cassette which comprises a tissue plasminogen

activator leader sequence fused to a codon-optimized gag gene which is under the control of the CMV promoter and contains Intron A.

"FI" refers to a full length gene.

"Flgag" refers to the full-length optimized gag gene.

5 "Ad5-Flgag" refers to an adenovirus serotype 5 replication deficient virus which carries an expression cassette which comprises a full length optimized gag gene under the control of the CMV promoter and contains Intron A.

"FG Adenovirus" means a First Generation adenovirus, i.e. a replication deficient adenovirus which has either a non-functional or deleted E1
10 region, and optionally a non-functional or deleted E3 region.

"Promoter" means a recognition site on a DNA strand to which an RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences such as enhancers or inhibiting sequences such as silencers.

15 "Leader" means a DNA sequence at the 5' end of a structural gene which is transcribed along with the gene. This usually results a protein having an N-terminal peptide extension, often referred to as a pro-sequences.

"Intron" as used herein refers to a section of DNA occurring in the middle of a gene which does not code for an amino acid in the gene product. The
20 precursor RNA of the intron is excised and is therefore not transcribed into mRNA not translated into protein.

"Cassette" refers to the a nucleic acid sequence which is to be expressed, along with its transcription and translational control sequences. By changing the cassette, a vector can express a different sequence.

25 It has been found according to this invention that first generation adenoviral vectors carrying a codon-optimized HIV gag gene regulated with a strong heterologous promoter can be used as human anti-HIV vaccines, and are capable of inducing immune responses.

The adenoviral vector which makes up the backbone of the vaccine
30 construct of this invention is preferably a "first generation" adenoviral vector. This group of adenoviral vectors is known in the art, and these viruses are characterized by being replication-defective. They typically have a deleted or inactivated E1 gene region, and preferably additionally have a deleted or inactivated E3 gene region. In a preferred embodiment of this invention, the first generation replication incompetent
35 adenovirus vector used is a serotype 5 adenovirus containing deletions in E1 (Ad5

base pairs 342-3523) and E3 (Ad5 base pairs 28133 to 30818).. For adenovirus 2 serotype, the E1 deletions are preferably bp 559-3503 and the E3 deletions are preferably 28,812-29,773. (Genbank gb:J01917). Those of skill in the art can easily determine the equivalent sequences for other serotypes, such as serotypes 4, 12, 6, 17, 24, 33, 42, 31, 16.

Adenoviral serotypes 2 and 5, particularly 5 are preferred for use in this invention, since at this point in time, more is known about these serotypes generally than other serotypes, and their complete DNA sequences are known. The prototype serotype 5 adenovirus has been completely sequenced (Chroboczek et al, 1992 *J. Virology* 186:280, which is hereby incorporated by reference.) They also belong to the subgroup C adenoviruses, which are not associated with human or rodent malignancies. However, it is envisioned that any adenovirus serotype can be used in this invention, including non-human ones, as deletion of E1 genes should render all adenoviruses non-tumorigenic. Also it may be advantageous to use a serotype which has less prevalence in the wild, as patients are less likely to have previous exposure (and less pre-existing antibodies) to a rarer serotype.

The adenoviral vectors can be constructed using known techniques, such as those reviewed in Hitt et al, 1997 "Human Adenovirus Vectors for Gene Transfer into Mammalian Cells" *Advances in Pharmacology* 40:137-206, which is hereby incorporated by reference.

In constructing the adenoviral vectors of this invention, it is often convenient to insert them in to a plasmid or shuttle vector. These techniques are known and described in Hitt et al *supra*. This invention specifically includes both the adenovirus and the adenovirus when inserted into a shuttle plasmid.

Viral vectors can be propagated in various E1 complementing cell lines, including the known cell lines 293 and PER.C6. Both these cell lines express the adenoviral E1 gene product. PER.C6 is described in WO 97/00326, published January 3, 1997, which is hereby incorporated by reference. It is a primary human retinoblast cell line transduced with an E1 gene segment that complements the production of replication deficient (FG) adenovirus, but is designed to prevent generation of replication competent adenovirus by homologous recombination. 293 cells are described in Graham et al 1977 *J. Gen. Virol* 36:59-72, which is hereby incorporated by reference.

The HIV gag gene selected to be expressed is of importance to the invention. Sequences for many genes of many HIV strains are publicly available in

GENBANK and primary, field isolates of HIV are available from the National Institute of Allergy and Infectious Diseases (NIAID) which has contracted with Quality Biological (Gaithersburg, MD) to make these strains available. Strains are also available from the World Health Organization (WHO), Geneva Switzerland. In a preferred embodiment of this invention, the gag gene is from an HIV-1 strain (CAM-1; Myers et al, eds. "Human Retroviruses and AIDS: 1995, IIA3-IIA19, which is incorporated by reference). This gene closely resembles the consensus amino acid sequence for the clade B (North American/European) sequence.

Regardless of the HIV gene chosen for expression, the sequence should be "optimized" for expression in a human cellular environment. A "triplet" codon of four possible nucleotide bases can exist in 64 variant forms. That these forms provide the message for only 20 different amino acids (as well as transcription initiation and termination) means that some amino acids can be coded for by more than one codon. Indeed, some amino acids have as many as six "redundant", alternative codons while some others have a single, required codon. For reasons not completely understood, alternative codons are not at all uniformly present in the endogenous DNA of differing types of cells and there appears to exist variable natural hierarchy or "preference" for certain codons in certain types of cells. As one example, the amino acid leucine is specified by any of six DNA codons including CTA, CTC, CTG, CTT, TTA, and TTG (which correspond, respectively, to the mRNA codons, CUA, CUC, CUG, CUU, UUA and UUG). Exhaustive analysis of genome codon frequencies for microorganisms has revealed endogenous DNA of *E. coli* most commonly contains the CTG leucine-specifying codon, while the DNA of yeasts and slime molds most commonly includes a TTA leucine-specifying codon. In view of this hierarchy, it is generally held that the likelihood of obtaining high levels of expression of a leucine-rich polypeptide by an *E. coli* host will depend to some extent on the frequency of codon use. For example, a gene rich in TTA codons will in all probability be poorly expressed in *E. coli*, whereas a CTG rich gene will probably highly express the polypeptide. Similarly, when yeast cells are the projected transformation host cells for expression of a leucine-rich polypeptide, a preferred codon for use in an inserted DNA would be TTA.

The implications of codon preference phenomena on recombinant DNA techniques are manifest, and the phenomenon may serve to explain many prior failures to achieve high expression levels of exogenous genes in successfully transformed host organisms--a less "preferred" codon may be repeatedly present in the

inserted gene and the host cell machinery for expression may not operate as efficiently. This phenomenon suggests that synthetic genes which have been designed to include a projected host cell's preferred codons provide a preferred form of foreign genetic material for practice of recombinant DNA techniques. Thus, one aspect of
5 this invention is an adenovirus vector which specifically includes a gag gene which is codon optimized for expression in a human cellular environment.

The diversity of function that typifies eukaryotic cells depends upon the structural differentiation of their membrane boundaries. To generate and maintain these structures, proteins must be transported from their site of synthesis in the
10 endoplasmic reticulum to predetermined destinations throughout the cell. This requires that the trafficking proteins display sorting signals that are recognized by the molecular machinery responsible for route selection located at the access points to the main trafficking pathways. Sorting decisions for most proteins need to be made only once as they traverse their biosynthetic pathways since their final destination, the
15 cellular location at which they perform their function, becomes their permanent residence.

Maintenance of intracellular integrity depends in part on the selective sorting and accurate transport of proteins to their correct destinations. Over the past few years the dissection of the molecular machinery for targeting and localization of
20 proteins has been studied vigorously. Defined sequence motifs have been identified on proteins which can act as "address labels". Leader or signal peptides such as that from the tissue-specific plasminogen activator protein, tPA, serve to transport a protein into the cellular secretory pathway through the endoplasmic reticulum and golgi apparatus. A number of sorting signals have been found associated with the
25 cytoplasmic domains of membrane proteins such as di-Leucine amino acid motifs or tyrosine-based sequences that can direct proteins to lysosomal compartments. For HIV, transport and extrusion from the cell of viral particles depend upon myristoylation of glycine residue number two at the amino terminus of gag. In some embodiments of the optimized gag gene, the tPA leader sequence has been attached 5'
30 to the structural gene sequence.

The optimized gag gene is incorporated into an expression cassette. The cassette contains a transcriptional promoter recognized by an eukaryotic RNA polymerase, and a transcriptional terminator at the end of the gag gene coding sequence. In a preferred embodiment, the promoter is a "strong" or "efficient"
35 promoter. An example of a strong promoter is the immediate early human

cytomegalovirus promoter (Chapman et al, 1991 *Nucl. Acids Res*19:3979-3986, which is incorporated by reference) with the intron A sequence (CMV-intA), although those skilled in the art will recognize that any of a number of other known promoters, such as the strong immunoglobulin, or other eukaryotic gene promoters may be used, including the EF1 alpha promoter, the murine CMV promoter, Rous sarcoma virus (RSV) promoter, SV40 early/late promoters and the beta-actin promoter. A preferred transcriptional terminator is the bovine growth hormone terminator. The combination of CMVintA-BGH terminator is particularly preferred although other promoter/terminator combinations in the context of FG adenovirus may also be used.

To assist in preparation of the polynucleotides in prokaryotic cells, a shuttle vector version of the adenovirus vector is often prepared. The shuttle vector contains an adenoviral portion and a plasmid portion. The adenoviral portion is essentially the same as the adenovirus vector discussed *supra*, containing adenoviral sequences (with non-functional or deleted E1 and E3 regions) and the gag expression cassette, flanked by convenient restriction sites. The plasmid portion of the shuttle vector often contains an antibiotic resistance marker under transcriptional control of a prokaryotic promoter so that expression of the antibiotic does not occur in eukaryotic cells. Ampicillin resistance genes, neomycin resistance genes and other pharmaceutically acceptable antibiotic resistance markers may be used. To aid in the high level production of the polynucleotide by fermentation in prokaryotic organisms, it is advantageous for the shuttle vector to contain a prokaryotic origin of replication and be of high copy number. A number of commercially available prokaryotic cloning vectors provide these benefits. It is desirable to remove non-essential DNA sequences. It is also desirable that the vectors not be able to replicate in eukaryotic cells. This minimizes the risk of integration of polynucleotide vaccine sequences into the recipients' genome. Tissue-specific promoters or enhancers may be used whenever it is desirable to limit expression of the polynucleotide to a particular tissue type.

In one embodiment of this invention, the shuttle plasmid used is pAD.CMVI-FLHIVgag, was made using homologous recombination techniques. For clinical use, the shuttle vector was rescued into virus in PER.C6 cells. To rescue, the shuttle plasmid was linearized by *PacI* restriction enzyme digestion and transfected into the PER.C6 cells using the calcium phosphate coprecipitate method. The plasmid in linear form is capable of replication after entering the PER.C6 cells and

virus is produced. The infected cells and media were harvested after viral replication was complete.

Standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of the DNA immunogens of this invention.

5 To ensure a clonal virus population a method of clonal purification was used for clinical material. The virus obtained from transfection of the PER.C6 cells was serially diluted to extinction using 2-fold dilutions. The dilutions were then used to infect PER.C6 cells in 96 well plates using 24 wells for each solution. At the end of a 14-day incubation period the wells were scored positive or negative using
10 adenovirus specific PCR and gag ELISA. Virus positive wells at the highest dilutions were selected for expansion. The selected well was the only positive well out of 24 wells plated at that dilution giving 98% assurance of clonality. Verification of that endpoint had been reached in the dilution series, and that virus positive wells that had insufficient virus to be detected in the initial screening had not been missed, was
15 obtained by subculturing the original 96 well plated two additional times and re-scoring them. This confirmed the clonality of the selected well. The selected virus was designated AD5FLgag.

The adenoviral vaccine composition may contain physiologically acceptable components, such as buffer, normal saline or phosphate buffered saline,
20 sucrose, other salts and polysorbate. One preferred formulation has: 2.5-10 mM TRIS buffer, preferably about 5 mM TRIS buffer; 25-100 mM NaCl, preferably about 75 mM NaCl; 2.5-10% sucrose, preferably about 5% sucrose; 0.01 -2 mM MgCl₂; and 0.001%-0.01% polysorbate 80 (plant derived). The pH should range from about 7.0-9.0, preferably about 8.0. One skilled in the art will appreciate that other conventional
25 vaccine excipients may also be used it make the formulation. The preferred formulation contains 5mM TRIS, 75 mM NaCl, 5% sucrose, 1mM MgCl₂, 0.005% polysorbate 80 at pH 8.0. This has a pH and divalent cation composition which is near the optimum for Ad5 stability and minimizes the potential for adsorption of virus to a glass surface. It does not cause tissue irritation upon intramuscular injection. It is
30 preferably frozen until use.

The amount of adenoviral particles in the vaccine composition to be introduced into a vaccine recipient will depend on the strength of the transcriptional and translational promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of 1×10^7
35 to 1×10^{12} particles and preferably about 1×10^{10} to 1×10^{11} particles is administered

directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated. It is also contemplated that booster vaccinations are to be provided. Following vaccination
5 with HIV adenoviral vector, boosting with a subsequent HIV adenoviral vector and/or plasmid may be desirable. Parenteral administration, such as intravenous, intramuscular, subcutaneous or other means of administration of interleukin-12 protein, concurrently with or subsequent to parenteral introduction of the vaccine compositions of this invention is also advantageous.

10 Another aspect of this invention is the administration of the adenoviral vector containing the optimized gag gene in a prime/boost regimen in conjunction with a plasmid DNA encoding gag. To distinguish this plasmid from the adenoviral-containing shuttle plasmids used in the construction of an adenovirus vector, this plasmid will be referred to as a "vaccine plasmid". The preferred vaccine plasmids to
15 use in this administration protocol are disclosed in pending U.S. patent application 09/017,981, filed February 3, 1998 and WO98/34640, published August 13, 1998, both of which are hereby incorporated by reference. Briefly, the preferred vaccine plasmid is designated VIJns-FL-gag, which expresses the same codon-optimized gag gene as the adenoviral vectors of this invention. The vaccine plasmid backbone,
20 designated VIJns contains the CMV immediate-early (IE) promoter and intron A, a bovine growth hormone-derived polyadenylation and transcriptional termination sequence as the gene expression regulatory elements, and a minimal pUC backbone (Montgomery et al, 1993 *DNA Cell Biol.* 12:777-783. The pUC sequence permits high levels of plasmid production in *E. coli* and has a neomycin resistance gene in
25 place of an ampicillin resistance gene to provide selected growth in the presence of kanamycin. Those of skill in the art, however, will recognize that alternative vaccine plasmid vectors may be easily substituted for this specific construct, and this invention specifically envisions the use of alternative plasmid DNA vaccine vectors.

The adenoviral vector and/or vaccine plasmids of this invention
30 polynucleotide may be unassociated with any proteins, adjuvants or other agents which impact on the recipients' immune system. In this case, it is desirable for the vector to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. Alternatively, the vector may be associated with an adjuvant known in the art to boost immune responses, such as a protein or other
35 carrier. Agents which assist in the cellular uptake of DNA, such as, but not limited to,

calcium ions, may also be used to advantage. These agents are generally referred to herein as transfection facilitating reagents and pharmaceutically acceptable carriers. Techniques for coating microprojectiles coated with polynucleotide are known in the art and are also useful in connection with this invention.

5 The adenoviral vaccines of this invention may be administered alone, or may be part of a prime and boost administration regimen. A mixed modality priming and booster inoculation scheme will result in an enhanced immune response, particularly if pre-existing anti-vector immune responses are present. This one aspect of this invention is a method of priming a subject with the plasmid vaccine by
10 administering the plasmid vaccine at least one time, allowing a predetermined length of time to pass, and then boosting by administering the adenoviral vaccine. Multiple primings typically, 1-4, are usually employed, although more may be used. The length of time between priming and boost may typically vary from about four months to a year, but other time frames may be used. In experiments with rhesus monkeys,
15 the animals were primed four times with plasmid vaccines, then were boosted 4 months later with the adenoviral vaccine. Their cellular immune response was notably higher than that of animals which had only received adenoviral vaccine. The use of a priming regimen may be particularly preferred in situations where a person has a pre-existing anti-adenovirus immune response.

20 This invention also includes a prime and boost regimen wherein a first adenoviral vector is administered, then a booster dose is given. The booster dose may be repeated at selected time intervals.

25 A large body of human and animal data supports the importance of cellular immune responses, especially CTL in controlling (or eliminating) HIV infection,. In humans, very high levels of CTL develop following primary infection and correlate with the control of viremia. Several small groups of individuals have been described who are repeatedly exposed to HIV by remain uninfected; CTL has been noted in several of these cohorts. In the SIV model of HIV infection, CTL
30 similarly develops following primary infection, and it has been demonstrated that addition of anti-CD8 monoclonal antibody abrogated this control of infection and leads to disease progression. This invention uses adenoviral vaccines alone or in combination with plasmid vaccines to induce CTL.

Cellular Immunity Assays for Pre-Clinical and Clinical Research

Another aspect of this invention is an assay which measures the elicitation of HIV-1 protein, including gag-specific cellular immunity, particularly cytotoxic T-lymphocyte (CTL) responses. The "ELISpot" and cytotoxicity assays, discussed herein, measure HIV antigen-specific CD8+ and CD4+ T lymphocyte responses, and can be used for a variety of mammals, such as humans, rhesus monkeys, mice, and rats.

The ELISpot assay provides a quantitative determination of HIV-specific T lymphocyte responses. PMBC cells are cultured in tissue culture microtiter plates. An HIV-1 gag peptide pool that encompasses the entire 500 amino acid open reading frame of gag (50 overlapping 20mer peptides) is added to the cells and one day later the number of cells producing gamma interferon (or another selected interferon) is measured. Gamma interferon was selected as the cytokine visualized in this assay (using species specific anti-gamma interferon monoclonal antibodies) because it is the most common, and one of the most abundant cytokines synthesized and secreted by activated T lymphocytes. For this assay, the number of spot forming cells (SPC) per million PBMCs is determined for samples in the presence and absence (media control) of peptide antigens. This assay may be set up to determine overall T lymphocyte responses (both CD8+ and CD4+) or for specific cell populations by prior depletion of either CD8+ or CD4+ T cells. In addition, ELISpot assays, or variations of it, can be used to determine which peptide epitopes are recognized by particular individuals.

A distinguishing effector function of T lymphocytes is the ability of subsets of this cell population to directly lyse cells exhibiting appropriate MHC-associated antigenic peptides. This cytotoxic activity is most often associated with CD8+ T lymphocytes but may also be exhibited by CD4+ T lymphocytes. We have optimized bulk culture CTL assays in which PBMC samples are infected with recombinant vaccinia viruses expressing antigens (e.g., gag) in vitro for approximately 14 days to provide antigen restimulation and expansion of memory T cells that are then tested for cytotoxicity against autologous B cell lines treated either with peptide antigen pools. Specific cytotoxicity is measured compared to irrelevant antigen or excipient-treated B cell lines. The phenotype of responding T lymphocytes is determined by appropriate depletion of either CD8+ or CD4+ populations prior to

the cytotoxicity assay. This assay is semi-quantitative and is the preferred means for determining whether CTL responses were elicited by the vaccine.

5 The following non-limiting Examples are presented to better illustrate the invention.

EXAMPLES

EXAMPLE 1

10 Construction of replication-defective
FG-Ad expressing HIV gag antigen

Starting vectors

15 Shuttle vector pHCMVIBGHpA1 contains Ad5 sequences from bp1 to bp 341 and bp 3534 to bp 5798 with a expression cassette containing human cytomegalovirus (HCMV) promoter plus intron A and bovine growth hormone polyadenylation signal.

20 The adenoviral backbone vector pAdE1-E3- (also named as pHVad1) contains all Ad5 sequences except those nucleotides encompassing the E1 and E3 region.

25 Plasmid pVIJNStpaHIVgag contains tPA secretory signal sequence fused to the codon-optimized HIV gag nucleotides under the control of HCMV promoter plus intron A. It is described in pending U.S. patent application 09/017,981, filed February 3, 1998 and WO98/34640, published August 13, 1998, both of which are hereby incorporated by reference.

Plasmid pVIR-FLHIV gag (also named as pVIR-HIVgag-opt) contains codon-optimized full-length HIV gag under the control of the HCMV promoter plus intron A.

30 Construction of Ad5.tpaHIVgag

1. Construction of adenoviral shuttle plasmid pA1-CMVI-tpaHIVgag containing tPAgag under the control of human CMV promoter and intron A.

35 The tPAgag insert was excised from pVIJNS-tPAgag by restriction enzymes PstI and XmaI, blunt-ended, and then cloned into EcoRV digested shuttle vector pHCMVIBGHpA1. The orientation of the transgene and the construct were

verified by PCR using the insert specific primers hCMV5'-4 (5' TAG CGG CGG AGC TTC TAC ATC 3' SEQ.ID.NO. __) and Gag3'-1 (5' ACT GGG AGG AGG GGT CGT TGC 3' SEQ.ID.NO. __), restriction enzyme analysis (RcaI, SspBI), and DNA sequencing spanning from CMV promoter to the initiation of the gag.

5

2. Homologous recombination to generate shuttle plasmid form of recombinant adenoviral vector pAd-CMVI-tpaHIVgag containing tpaHIVgag expression cassette.

Shuttle plasmid pA1-CMVI-tpaHIVgag was digested with restriction enzymes BstZ17 and SgrA1 and then co-transformed into *E. coli* strain BJ5183 with linearized (ClaI digested) adenoviral backbone plasmid pAdE1-E3-. One colony was verified by PCR analysis. The vector was transformed to competent *E. coli* HB101 for large quantity production of the plasmid.

3. Generation of recombinant adenovirus Ad.CMVI-tpaHIVgag in 293 cells.

The shuttle plasmid was linearized by restriction enzyme PacI and transfected to 293 cells using CaPO₄ method (InVitrogen kit). Ten days later, 10 plaques were picked and grown in 293 cells in 35-mm plates. PCR analysis of the adenoviral DNA indicated 10 out of 10 virus were positive for gag.

4. Evaluation of large scale recombinant adenovirus Ad.CMVI-tpaHIVgag

Clone No.9 was grown into large quantities through multiple rounds of amplification in 293 cells. One lot yielded of 1.7×10^{12} particles and a second lot yielded 6.7×10^{13} particles. The viral DNA was extracted by proteinase K digestion and confirmed by PCR and restriction enzyme (HindIII) analysis. The expression of tpaHIVgag was also verified by ELISA and Western blot analysis of the 293 or COS cells infected with the recombinant adenovirus. The recombinant adenovirus was used for evaluation in mice and rhesus monkeys.

Construction of Ad5.FHIVgag

1. Construction of adenoviral shuttle plasmid pA1-CMVI-FLHIVgag containing full length HIVgag under the control of human CMV promoter and intron A.

The FLHIVgag insert was excised from pVIR-FLHIVgag by restriction enzyme BglII and then cloned into BglII digested shuttle vector pHCMVIBGHpA1. The orientation and the construct were verified by PCR using the

insert specific primers (hCMV5'-4 and Gag3'-1), restriction enzyme analysis, and DNA sequencing.

2. Homologous recombination to generate plasmid form of recombinant adenoviral vector pAd-CMVI-FLHIVgag containing FLHIVgag expression cassette.

Shuttle plasmid pA1-CMVI-FLHIVgag was digested with restriction enzymes BstZ17 and SgrA1 and then co-transformed into *E. coli* strain BJ5183 with linearized (ClaI digested) adenoviral backbone plasmid pAdE1-E3-. Colonies #6 and #7 were verified by PCR analysis. The vectors were transformed to competent *E. coli* HB101 for large quantity production of the plasmid. The plasmids were verified by HindIII digestion.

3. Generation of recombinant adenovirus Ad.CMVI-FLHIVgag in 293 cells.

The pAd plasmids were linearized by restriction enzyme PacI and transfected to 293 cells using Lipofectamine (BRL). Two weeks later, 6 viruses (#6-1.1, 6-1.2, 6-1.3, 7-1.1, 7-1.2, 7-1.3) were picked and grown in 293 cells in 35-mm plates. PCR analysis using the insert specific primers (hCMV5'-4 and Gag3'-1) of the adenoviral DNA verified the presence of HIV gag.

4. Evaluation of large scale recombinant adenovirus Ad.CMVI-FHIVgag

Virus clone #6-1 was grown into large quantities through multiple rounds of amplification in 293 cells. The viral DNA was extracted by proteinase K digestion and confirmed by PCR, restriction enzyme (HindIII, Bgl II, Bst E II, Xho I) analysis. A partial sequencing confirmed the junction between CMV promoter and the 5' end of HIV gag gene. The expression of FLHIVgag was also verified by ELISA and Western blot analysis of the 293 or COS cells infected with the recombinant adenovirus. The recombinant adenovirus was used for evaluation in mice and rhesus monkeys.

Construction of FG adenovirus FL gag. The full-length (FL) humanized gag gene was ligated into an adenovirus-5 shuttle vector, pHCMVIBGHpA1, containing Ad5 sequences from bp 1 to bp 341 and bp 3534 to bp 5798 with a expression cassette containing human CMV promoter plus intron A and bovine growth hormone polyadenylation signal. The orientation was confirmed by restriction enzyme digestion analysis and DNA sequencing. Homologous recombination in *E. coli* was employed using the shuttle plasmid, pA1-CMVI-

FLHIVgag, and adenoviral backbone plasmid, pAdE1-E3-, to generate a plasmid form of the recombinant adenovirus containing the expression regulatory elements and FL gag gene, pAd.CMVI-FHIVgag. Appropriate plasmid recombinants were confirmed by restriction enzyme digestion.

5 The pAd plasmid containing the gag expression cassette was linearized by restriction enzyme PacI and transfected to 293 cells (or PER.C6 cells for clinical development candidates) using Lipofectamine (BRL). Two weeks later, 6 viruses were picked and grown in 293 cells in 35-mm plates. PCR analysis using the insert specific primers (hCMV5'-4 and Gag3'-1) of the adenoviral DNA verified the
10 presence of HIV gag. Virus clone #6-1 was grown into large quantities through multiple rounds of amplification in 293 cells. The viral DNA was extracted by proteinase K digestion and confirmed by PCR, restriction enzyme (HindIII, BglII, BstEII, XhoI) analysis. A partial sequencing confirmed the junction between CMV promoter and the 5' end of HIV gag gene. Restriction enzyme analysis demonstrated
15 that the viral genome was stable over the course of these passages.

 The expression of HIV gag was verified by ELISA and Western blot analysis of the 293 or COS cells infected with the recombinant adenovirus.

EXAMPLE 2

20 Immunogenicity/Preclinical Efficacy

The "ELISpot" Assay

 The ELISpot assay is a quantitative determination of IV-specific T lymphocyte responses by visualization of gamma interferon secreting cells in tissue
25 culture microtiter plates one day following addition of an HIV-1 gag peptide pool that encompasses the entire 500 amino acid open reading frame of gag (50 overlapping 20mer peptides) to PBMC samples. The number of spot forming cells (SPC) per million of PBMCs is determined for samples in the presence and absence (media control) of peptide antigens. The assay may be set up to determine overall T
30 lymphocyte responses (both CD8+ and CD4+) or for specific cell populations by prior depletion of either CD8+ or CD4+ cells. In addition, the assay can be varied so as to determine which peptide epitopes are recognized by particular individuals.

Cytotoxic T Lymphocyte Assays

 In this assay, PBMC samples are infected with recombinant vaccinia viruses
35 expressing gag antigen *in vitro* for approximately 14 days to provide antigen

restimulation and expansion of memory T cells. The cells are then tested for cytotoxicity against autologous B cell lines treated with peptide antigen pools. The phenotype of responding T lymphocytes is determined by appropriate depletion of either CD8+ or CD4+ cells.

5 A. Immune Responses to FG Adenovirus 5 FLgag Vaccine in Rodents

Adenovirus vectors coding for the gag antigen have consistently produced significantly stronger cellular immune responses than plasmid vectors in rodent species. Table 1 (below) shows ELISpot data from mice vaccinated with Ad5FLgag in comparison with plasmid DNA. Splens from five mice were pooled
10 and the number of gag peptide-specific interferon-gamma secreting cells was determined.

Table 1: Comparison of plasmid and adenovirus vaccination in mice

	SFC/10 ⁶ splenocytes	
	Post 1st Vaccination	Post 2nd Vaccination
10 µg plasmid	68	324
10 ⁵ Ad5FLgag	18	170
10 ⁸ Ad5FLgag	530	5600

Similar enhancements in the cellular responses to gag were also seen in Fischer rats. FIGURE 1 shows the ELISpot data from individual rats vaccinated with 10¹⁰ particles of adenovirus Ad5FLgag or with 0.4 mg FL gag plasmid. The mean
20 response after one vaccination was 10-fold higher with adenovirus compared to plasmid. Both vaccines gave a boosted signal after a second vaccination, with the adenovirus vaccine signal 5-fold higher than the plasmid signal.

25 B. Immune Responses to FG Adenovirus 5 FL gag Vaccine in Rhesus Monkeys

Comparative in vivo expression of DNA vs. FGAd5 encoding a reporter gene. Adenovirus and plasmid vectors expressing the secreted alkaline phosphatase (SEAP) as a reporter gene were injected into rhesus monkeys to compare the levels of antigen produced by the two forms of vaccination as shown below.

FIGURE 2 shows that at the highest possible plasmid dose (5 mg), the antigen levels are 1,000-fold lower than the levels achieved using 10^{10} particles of adenovirus, a dose which is ten fold lower than the maximum proposed clinical dose.

5 FG adenovirus-5 FLgag vaccinations of rhesus monkeys. Three Rhesus monkeys were vaccinated at 0, 8, and 24 weeks with 10^{11} particles of FG adenovirus-5tPAgag, an adenoviral vector containing a form of the gag gene with a leader peptide from the tissue-specific plasminogen activator gene at the amino terminus. Data were collected starting at 20 weeks.

10 FIGURE 3 shows that all three monkeys had developed strong bulk culture cytotoxicity responses against gag peptide sensitized autologous B cell lines following *in vitro* restimulation using vaccinia-gag for two weeks. These responses were persistent at all time points tested although it is unclear whether the final inoculation at 24 weeks improved the cytotoxicities. In all cases killing is observed
15 with at least one partial peptide pool (i.e. 25 peptides from amino terminus, 25 peptides from the carboxyterminus of gag). In every case killing is observed with the full peptide pool (all 50 peptides spanning the full-length gag). ELIspot assays showed high levels of gamma interferon secreting cells (approximately 200-1000 SFC/million PBMCs) over the course of these experiments, and CD4/8 depletion
20 studies indicated that most responding cells were CD8 T lymphocytes, although most vaccines also had significant CD4 T cell responses.

Immunization of rhesus monkeys with FG adenovirus-5 FLgag. Using a protocol similar to that described above, monkeys were vaccinated with a FG adenovirus-5 construct encoding a full-length gag gene (without the tPA leader
25 peptide). This experiment compared a dose titration of vaccine as well as needle vs. biojector (a needleless injector) delivery at most doses. A third feature of this experiment addresses the concerns raised above about the possible negative effects that prior adenovirus immunity may have on adenoviral vector mediated vaccine responses.

30 While approximately 150 rhesus sera have been tested for anti-adenovirus-5 neutralizing antibody responses, no significant titers have been detected. Rhesus are a poor host for this viral strain, while about 40-60% of humans have significant neutralizing antibody responses (titers from 10-500). For this experiment, two groups of monkeys (6 and 7) were pre-exposed to FG adenovirus-SEAP vectors

thrice and once, respectively, generating a range of neutralizing antibody responses in these monkeys that encompass the range observed in humans.

FIGURES 4A-I show bulk culture cytotoxicity responses of these vaccines at 8 weeks post a single immunization. All monkeys (groups 1-5, Figures 4A-E) that had not been previously exposed to adenovirus-5 showed significant gag-specific cytotoxic responses at all doses using either biojector or needle while 5/6 vaccines that had been preexposed to adenovirus showed cytotoxic responses (groups 6-7, Figure 4F-G). Control animals have remained consistently negative in these assays (e.g., group 8, Figure 4H).

Anti-gag ELISpot responses were also measured in all monkeys at eight weeks. Table 2, below is a summary of these responses that show that nearly all vaccines developed significant gamma-interferon responses to this vaccine, although prior exposure to adenovirus reduced response levels, and a dose response appears to have been obtained with the highest doses giving the best responses. In addition, in this experiment (as well as an independent experiment) no difference was observed for needle vs. biojector delivery of vaccine. CD4 T cell depletion of these samples showed that the ELISpot responses are largely due to CD8 T cells.

Table 2. Anti-gag ELISpot Responses of Rhesus Monkeys Immunized with FG adenovirus FLgag vaccine.

<u>Group</u>	<u>Rhesus #</u>	<u>Injection</u>	<u>Prior Adeno Exposure</u>	<u>SFC/million PBMCs</u>	
				<u>media</u>	<u>gag pool H</u>
1	96R044	Biojector	none	6	663
"	96R045	"	"	0	665
"	96R046	"	"	5	893
2	96R047	Biojector	none	1	20
"	96R048	"	"	1	104
"	96R049	"	"	0	38
3	96R050	Biojector	none	4	18
"	96R051	"	"	1	14
"	96R052	"	"	10	48
4	96R053	Needle	none	1	410

"	96R054	"	"	0	125
"	96R057	"	"	3	186
5	96R058	Needle	none	1	93
"	96R060	"	"	1	41
"	96R062	"	"	0	6
6	940125	Biojector	3X 10 ¹⁰ FG aden-5	15	65
"	940132	"	"	11	39
"	940149	"	"	29	93
7	940145	Biojector	1X 10 ¹⁰ FG adeno-5	4	258
"	940147	"	"	15	578
"	940217	"	"	23	55
8	96R063	none	none	0	0
"	96R004	"	"	0	1

These and other data show that higher doses of FG adenovirus vaccines elicited ELISpot responses as high as 800-1000 SFC/million PBMCs (see Table 2).

These responses are approximate 5-10 fold higher than those obtained using the highest doses of DNA gag vaccines after four injections over a six month time frame (see Table 3 below) indicating that FG adenovirus vaccines are much more potent than DNA vaccines. Importantly, these data also support the finding that repeated injection of adenoviral vector remain effective although somewhat attenuated in the presence of host immune response to adenovirus.

10 Combined DNA and FG adenovirus vaccinations in rhesus.

DNA priming may enhance the cellular immune response to gag induced by adenovirus vaccination as shown below. Three rhesus monkeys which had been vaccinated four times with 1 mg of gag plasmid were boosted 4 months following the final DNA shot with 10¹¹ particles of FG Ad FLgag. The cellular immune responses (measured by ELISpot and denoted as SFC/million PBMCs) to gag peptides in the monkeys primed with DNA and boosted with adenovirus appear significantly higher than adenovirus vaccination alone. The use of a DNA priming regimen may be particularly advantageous in humans who have preexisting anti-adenovirus immune responses.

Table 3. ELIspot Responses in Rhesus Monkeys After Combined DNA and FG adenovirus gag Vaccinations.

	Monkey #	Priming	Vaccine	Week 20 (2 injections)	Week 28 (3 injections)
DNA prime/	92x004	DNA	Ad5tPAgag	106	781
Ad boost	93x027	DNA	Ad5tPAgag	88	660
	93x023	DNA	Ad5tPAgag	560	609
DNA prime/	93x008	DNA	DNA	344	285
DNA boost	93x012	DNA	DNA	NA	NA
	93x016	DNA	DNA	106	99
Naive/Ad	92x024	None	Ad5tPAgag	373	898
	92x012	None	Ad5tPAgag	276	413
	94x025	None	Ad5tPAgag	531	1275
Control	088R	None	None	5	84
	115Q	None	None	0	8

Boost were performed at week 0, 8, and 24.

5

Table 4. Anti-gag antibody titer (mMU/ml) in Rhesus Monkeys After DNA and FG adenovirus gag Vaccinations.

	Monkey #	Week 0	Week 8 (1 injection)	Week 20 (2 injections)	Week 28 (3 injections)	Week 40
DNA prime/	92x004	25	7616	10133	12170	15892
Ad boost	93x027	114	36666	20523	95114	31437
	93x023	41	11804	12485	38579	17422
DNA prime/	93x008	158	1689	817	3882	1626
DNA boost	93x012	<10	512	216	722	132
	93x016	20	305	451	2731	735
Naive/Ad	92x024	<10	2454	11460	15711	7449
	92x012	<10	2161	5154	27029	8856
	94x025	14	5852	19159	45990	37586

Boost were performed at week 0, 8, and 24.

C. Determination of HIV-Specific T Lymphocyte Responses in HIV+ Humans

In order to qualify the CTL assays, PBMCs from HIV-1-infected patients, classified as long-term nonprogressors (LTNPs) due to their ability to maintain low levels of systemic viremia and high CD4⁺ T cell counts over a period of years, were used to measure systemic specific CTL responses. As discussed above, several studies have reported that the presence of HIV-1-specific CTL responses in infected individuals appears to correlate well with maintenance of disease-free infection.

Over the course of numerous independent experiments using PBMCs obtained from approximately 40 LTNP at five different clinical centers, these HIV-infected individuals generally exhibited strong gag-specific ELISpot and cytotoxicity responses that are predominantly mediated by CD8⁺ T lymphocytes (CD4⁺ responses are typically extremely low or undetectable in HIV+ individuals). The overall gag-specific ELISpot responses determined in these experiments are summarized below:

HIV+ ELISpot Response Summary

mean (\pm SD) SFC/million PBMCs (+ gag peptides) = 980 ± 1584

mean (\pm SD) SFC/million PBMCs (media control) = 24 ± 21

Similar experiments using PBMCs from 16 HIV seronegative individuals did not show significant gag-specific ELISpot or cytotoxicity responses. These ELISpot responses are summarized below:

HIV Seronegative ELISpot Response Summary

mean (\pm SD) SFC/million PBMCs (+ gag peptides) = 19 ± 28

mean (\pm SD) SFC/million PBMCs (media control) = 10 ± 14

The ELISpot assay provides a quantitative determination of HIV-specific T lymphocyte responses by visualization of gamma interferon-secreting cells in tissue culture microtiter plates one day following addition of an HIV-1 gag peptide pool that encompasses the entire 500 amino acid open reading frame of gag (50 overlapping 20mer peptides) to PBMC samples. Gamma interferon was selected as the cytokine visualized in this assay (using species specific anti-gamma interferon monoclonal antibodies) because it is the most common, and one of the most abundant

cytokines synthesized and secreted by activated T lymphocytes. For this assay, the number of spot forming cells (SPC) per million PBMCs is determined for samples in the presence and absence (media control) of peptide antigens. This assay may be set up to determine overall T lymphocyte responses (both CD8+ and CD4+) or for
5 specific cell populations by prior depletion of either CD8+ or CD4+ T cells. In addition, ELIspot assays, or variations of it, can be used to determine which peptide epitopes are recognized by particular individuals.

A distinguishing effector function of T lymphocytes is the ability of subsets of this cell population to directly lyse cells exhibiting appropriate MHC-
10 associated antigenic peptides. This cytotoxic activity is most often associated with CD8+ T lymphocytes but may also be exhibited by CD4+ T lymphocytes. We have optimized bulk culture CTL assays in which PBMC samples are infected with recombinant vaccinia viruses expressing antigens (e.g., gag) in vitro for approximately 14 days to provide antigen restimulation and expansion of memory T
15 cells that are then tested for cytotoxicity against autologous B cell lines treated either with peptide antigen pools. Specific cytotoxicity is measured compared to irrelevant antigen or excipient-treated B cell lines. The phenotype of responding T lymphocytes is determined by appropriate depletion of either CD8+ or CD4+ populations prior to the cytotoxicity assay. This assay is the best means for determining whether CTL
20 responses were elicited by the vaccine.

EXAMPLE 3

Clinical Trials

25 The safety and efficacy of a first generation adenovirus type 5 carrying an optimized gag gene alone and as part of a prime/boost protocol with a gag DNA plasmid are tested.

In the initial trial, subjects receive either 1mg or 5mg HIV gag DNA on a 0, 1, 2 month schedule. Equal number so of Ad5 seropositive and seronegative
30 subjects are involved in the study.

In a second trial, Ad5 seropositive and seronegative individuals receive either 10^7 or 10^9 particles per dose on a 0, 6 month schedule. Some of the individuals who have received a single dose of 10^9 particles of Ad5gag will also receive three injections of HIV gag DNA with 10^{11} particles of Ad5 gag. Also,

individuals who are Ad5 seropositive and seronegative naive individuals will receive 10^{11} particles on a 0, 6 month schedule.

- Safety and immunogenicity parameters: Each individual is bled for serum prior to day 0 to determine Ad5 neutralization titers and for PBMCs to establish B-LCL lines for bulk CTL determinations. On day 0 and 4 weeks following each dose of Ad5 gag, PBMCs will be drawn to determine CTL using bulk CTL and ELISPOT assays. Immunogenicity will also be measured at later time points to assess persistence of response.

Clinical Study Design Summary

- 10 1. Phase I study of HIV gag DNA Priming (plasmid only)

GROUP	Vaccine (n)	Placebo (n)	DNA dose	DNA regimen	Ad5 Sero-status
1	18	3	1 mg	0, 1, 2	+
2	18	3	1 mg	0, 1, 2	-
3	18	3	5 mg	0, 1, 2	+
4	18	3	5 mg	0, 1, 2	=
TOTAL	72	12			

2. Dose ranging study of AD5 gag

Group	Vaccine (n)	Placebo (n)	Ad5 Dose	Ad5 sero status
1	8	2	10^7	+
2	8	2	10^7	-
3	8	2	10^9	+
4	8	2	10^9	-
5	15	2	10^{11}	+
6	15	2	10^{11}	-
TOTAL	62	12		

15

3. Boosting of gag DNA by Ad5 gag

Group	Vaccine	Placebo	Ad5 dose	Ad5 sero-status	DNA dose	DNA regimen
1*	15	2	10 ¹¹	+	1 mg	0, 1, 2
2*	15	2	10 ¹¹	-	1 mg	0, 1, 2
3*	15	2	10 ¹¹	+	5 mg	0, 1, 2
4*	15	2	10 ¹¹	-	5 mg	0, 1, 2
	60	8				

* represent the same subjects from Phase 1 study, above.

WHAT IS CLAIMED IS:

1. A vaccine composition comprising a replication defective adenoviral vector comprising at least one gene encoding a HIV gag protein which is codon optimized for expression in a human, and the gene is operably linked to a heterologous promoter and transcription terminator.
2. An adenoviral vaccine vector comprising:
 - a) a replication defective adenoviral vector, wherein the adenoviral vector does not have a functional E1 gene, and further comprising:
 - b) a gene expression cassette comprising:
 - i) a nucleic acid encoding a gag protein which is codon optimized for expression in a human host;
 - ii) a heterologous promoter operatively linked to the nucleic acid encoding the gag protein; and
 - iii) a transcription terminator .
3. A vector according to Claim 2, wherein the E1 gene has been deleted from the adenoviral vector.
4. A vector according to Claim 3, wherein the gene expression cassette has replaced the deleted E1 gene.
5. A vector according to Claim 3, wherein the adenovirus vector does not have a functional E3 gene.
6. A vector according to Claim 5 wherein the E3 gene has been deleted from the replication defective adenoviral vector.
7. A vector according to Claim 6 comprising adenoviral 5 sequences deleted of E1 region base pairs (bp) 342-3523 and deleted of E3 region bp 28,133-30,818.
8. A vector according to Claim 6 comprising adenoviral 2 deleted of E1 region bp 559-3503 and E3 region bp 28,812-29,773.

9. A vector according to Claim 8 comprising the sequence given in
FIGURE 6.

5 10. A vector according to Claim 8 wherein the sequence is tPA-
gag.

10 11. A vector according to Claim 2 further comprising a
physiologically acceptable carrier.

12. An adenoviral vaccine composition for producing an immune
response against human immunodeficiency virus (HIV) in a human comprising:

- 15 a) adenovirus serotype 5 sequences bp 1 to bp 341 and bp 3534 to
5798; and
- b) a gene expression cassette, located 3' to adenovirus sequence bp
341, comprising:
- i) a nucleic acid encoding gag which is codon-optimized and
optionally has the tPA leader sequence at its 5' end;
- 20 ii) a human CMV promoter plus intron A operatively linked to
the nucleic acid encoding gag; and
- iii) a bovine growth hormone transcription terminator.

13. A plasmid vector comprising:

25 a) an adenoviral portion comprising an adenoviral vector according to
Claim 2; and

b) a plasmid portion.

14. A cell comprising an adenoviral vector of Claim 2.

30 15. A method of producing the vector of Claim 2 comprising
introducing the adenoviral vector of Claim 2 into a host cell which expresses
adenoviral E1 protein, and harvesting the resultant adenoviral vectors.

16. A method according to Claim 15 wherein the cell is a 293 cell or PER.C6 cell.

17. A method of generating a cellular immune response against an HIV protein in an individual comprising administering to the individual at least one adenovirus vaccine vector and a vaccine plasmid,
wherein said adenovirus vaccine vector comprises
a) a replication defective adenoviral vector, wherein the adenoviral vector does not have a functional E1 gene, and
b) a gene expression cassette comprising: i) a nucleic acid encoding gag protein optimized for expression in a human host; ii) a heterologous promoter operatively linked to the nucleic acid encoding the gag protein; and iii) a transcription terminator
wherein said vaccine plasmid comprises: i) a nucleic acid encoding a gag protein, wherein the nucleic acid is codon optimized for expression in a human host; ii) a promoter; and
c) a transcription terminator
wherein the vaccine plasmid does not contain any adenoviral genes.

18. A method according to Claim 17 comprising administering a vaccine plasmid to the individual, and after a predetermined minimum amount of time has passed, administering an adenovirus vaccine vector to the individual.

19. A method according to Claim 17 comprising administering an adenovirus vaccine vector to the individual, and after a predetermined minimum amount of time has passed, administering a vaccine plasmid to the individual.

20. A method according to Claim 14 comprising administering an adenovirus vaccine vector to the individual, and after a predetermined minimum amount of time has passed, re-administering an adenovirus vector to the individual.

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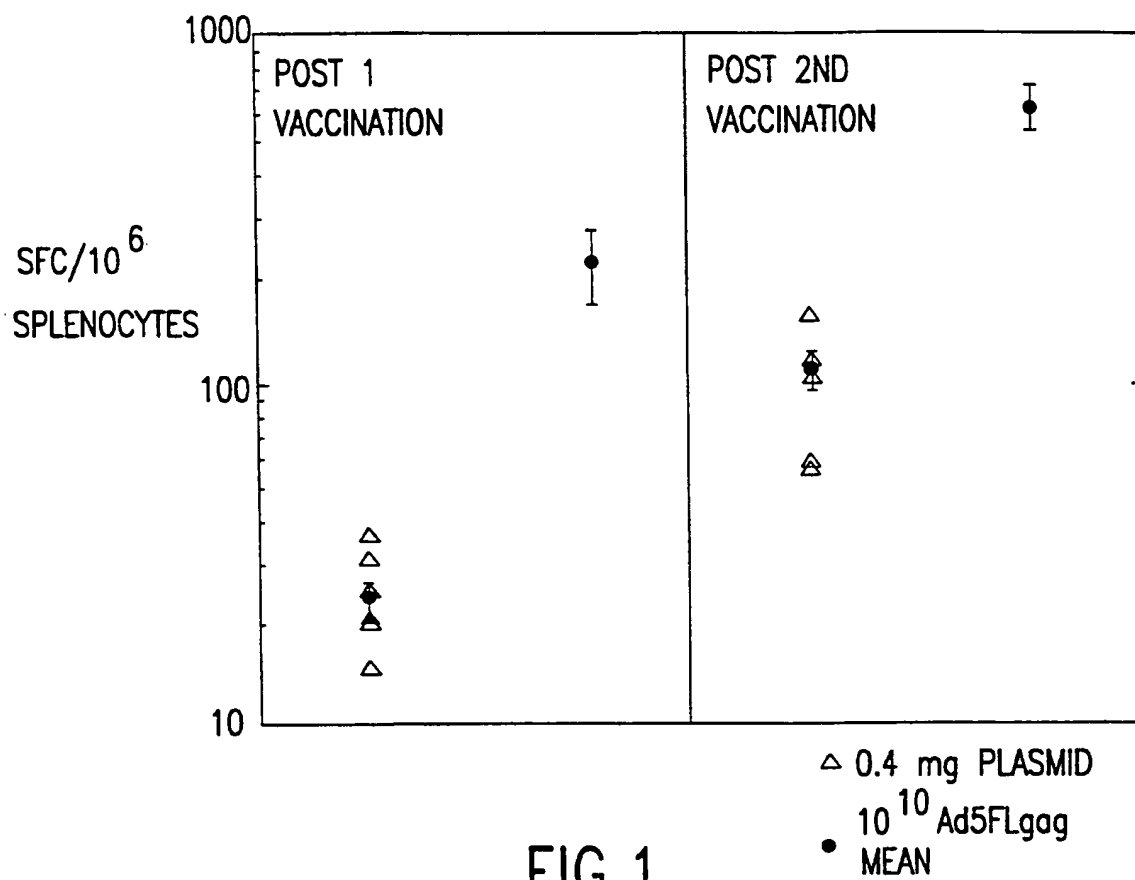


FIG.1

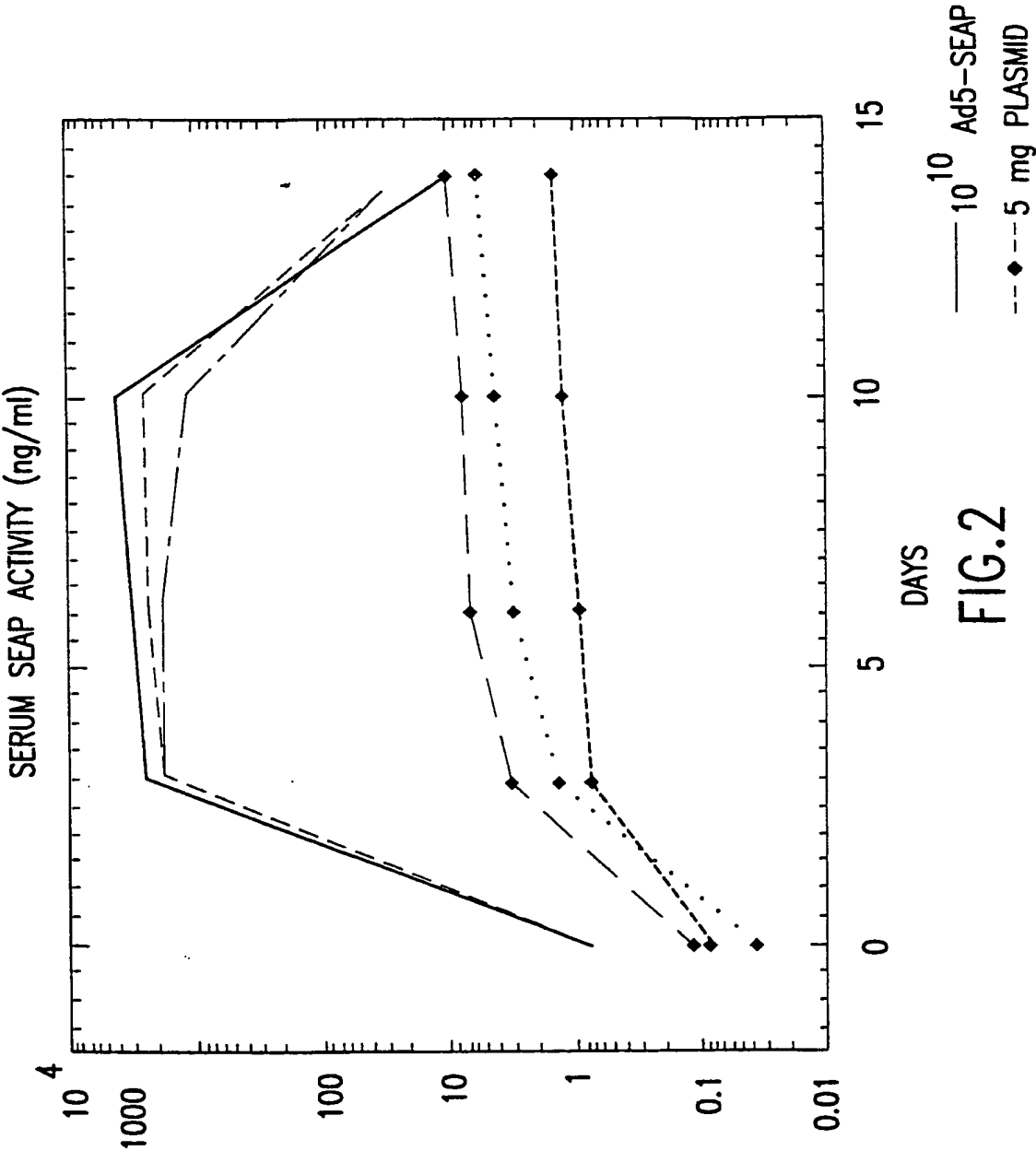
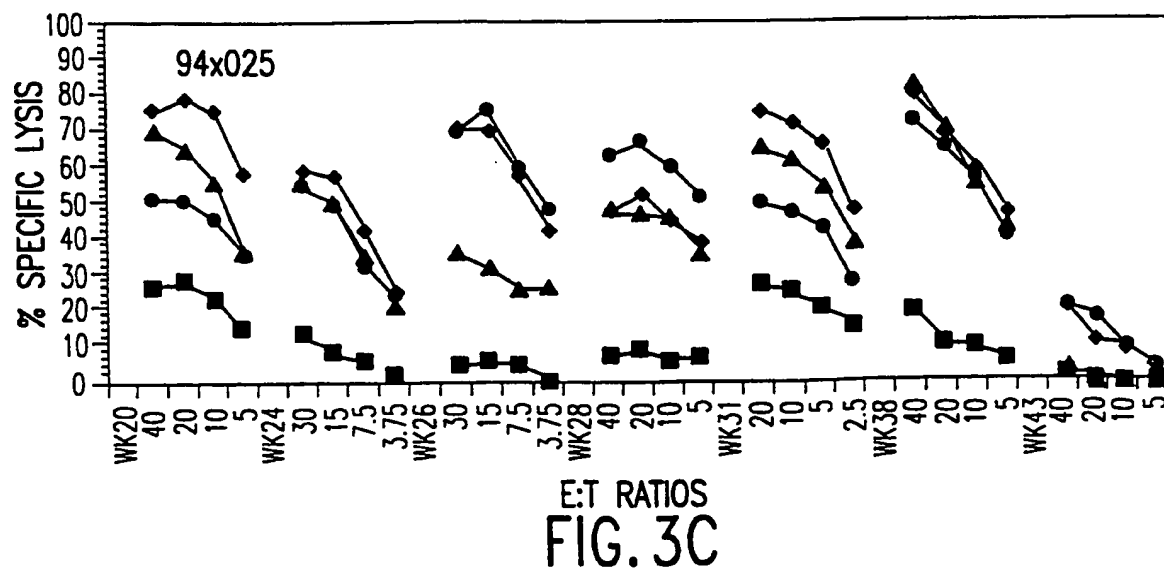
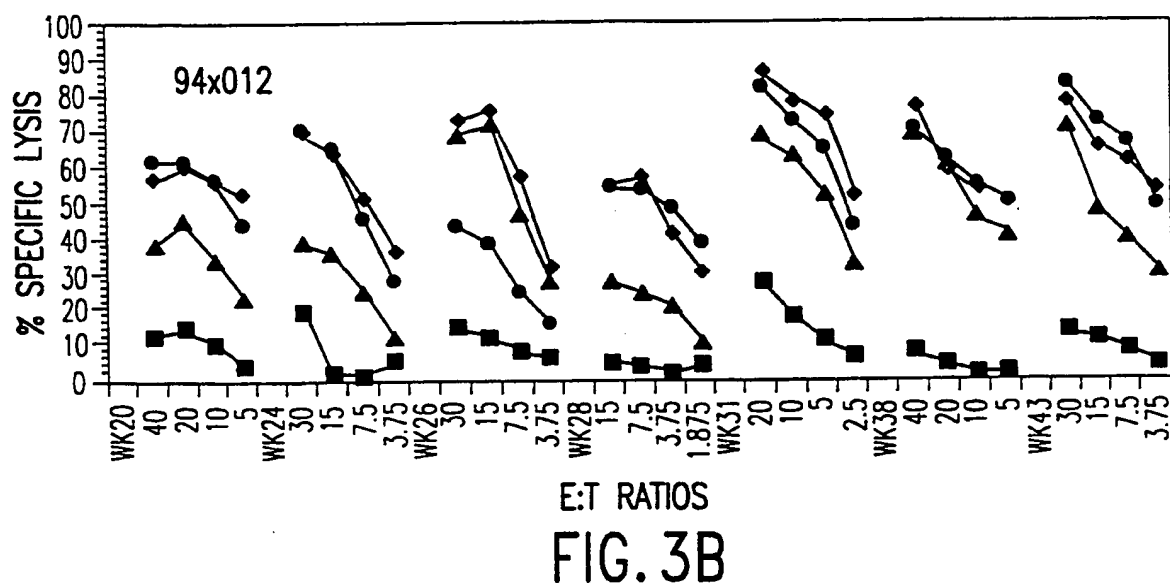
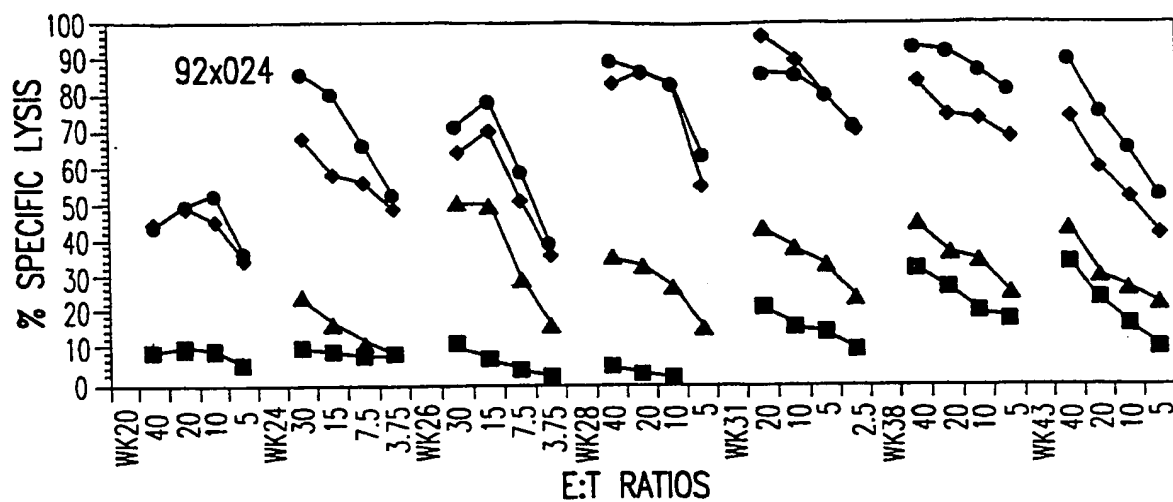
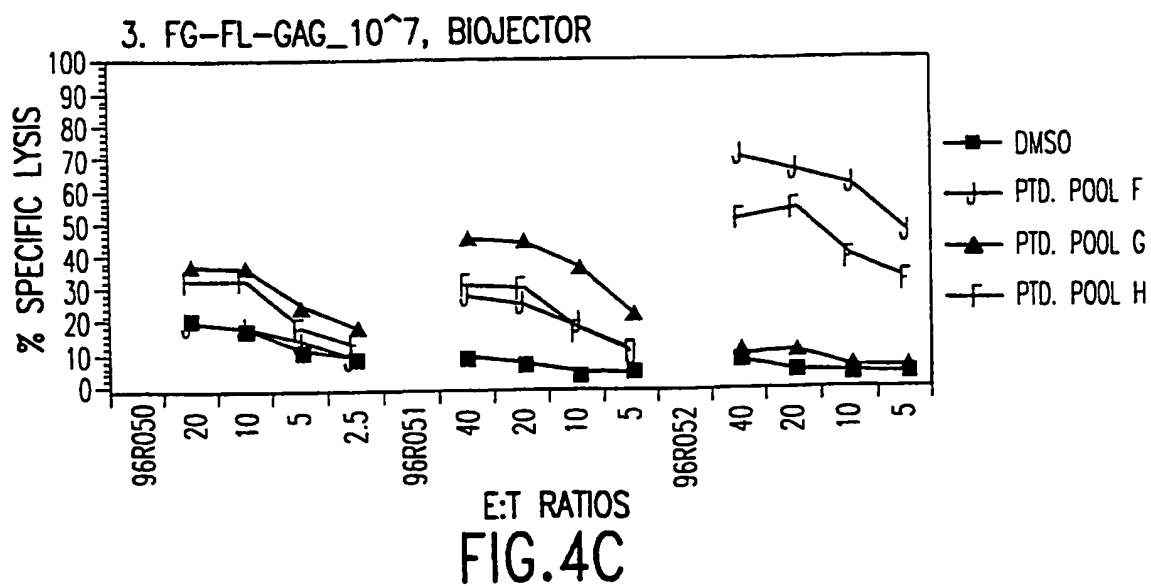
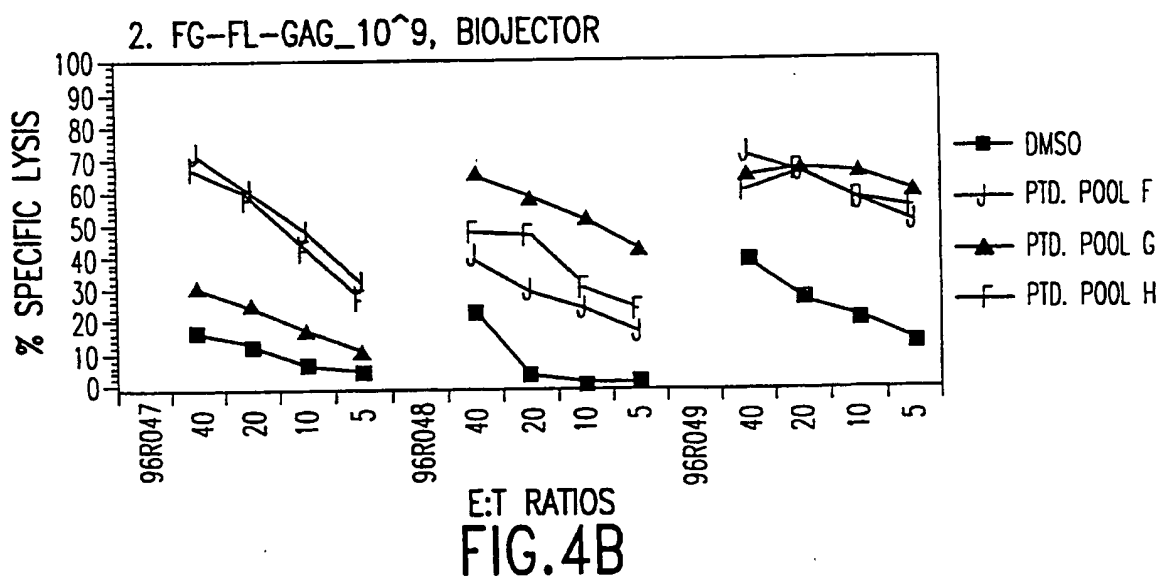
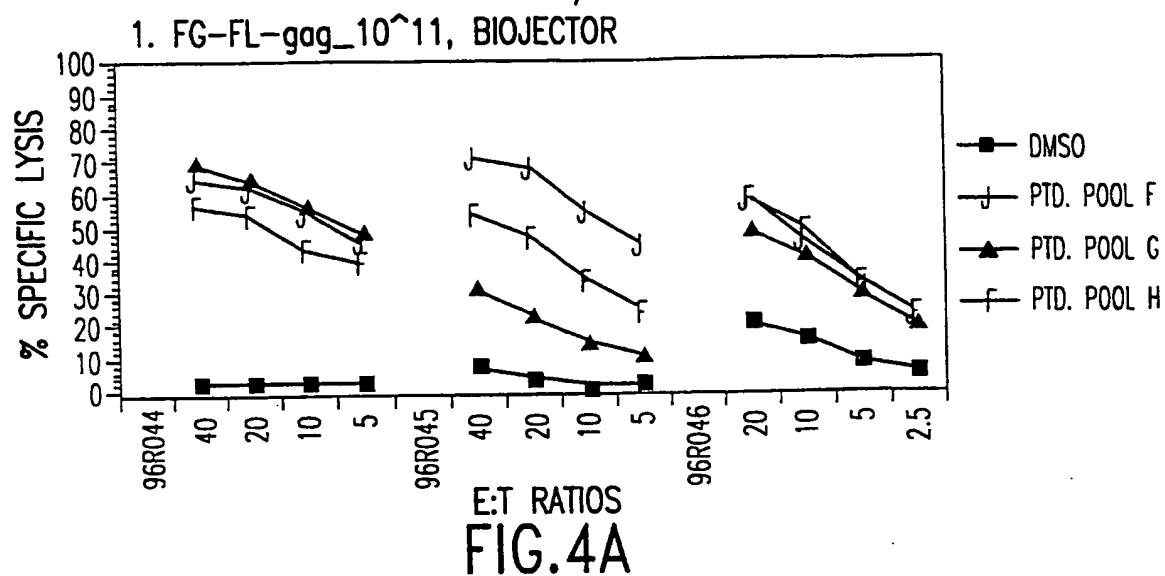


FIG.2

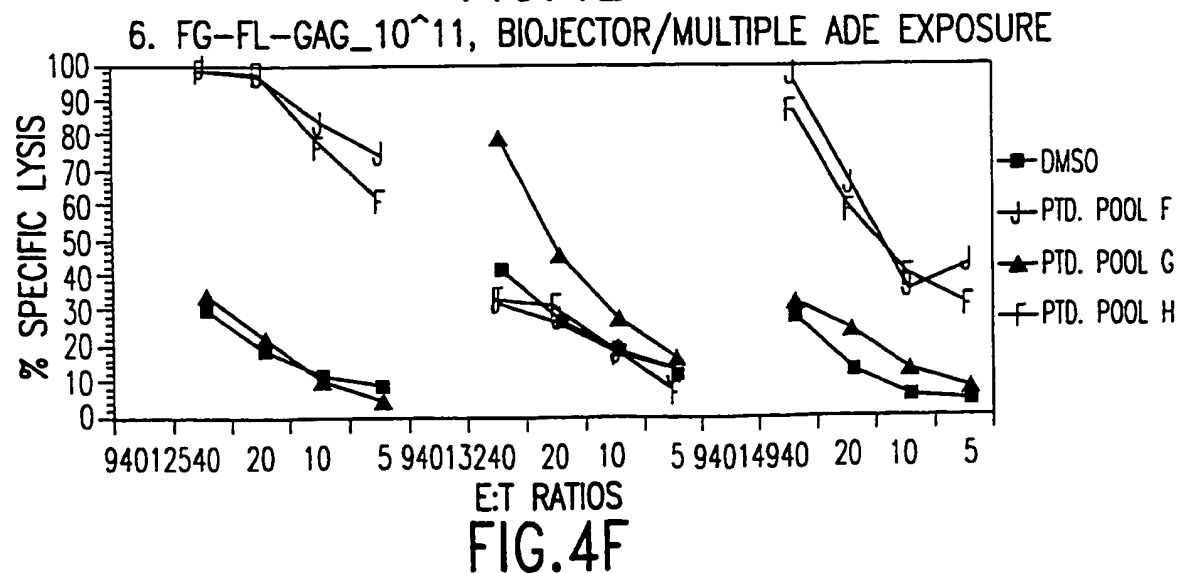
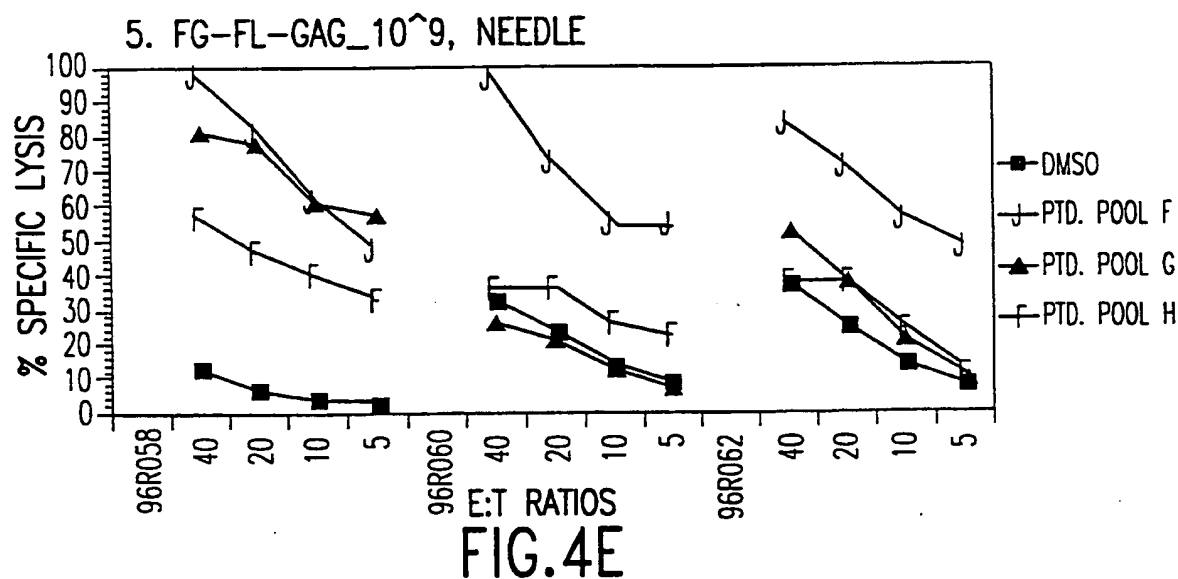
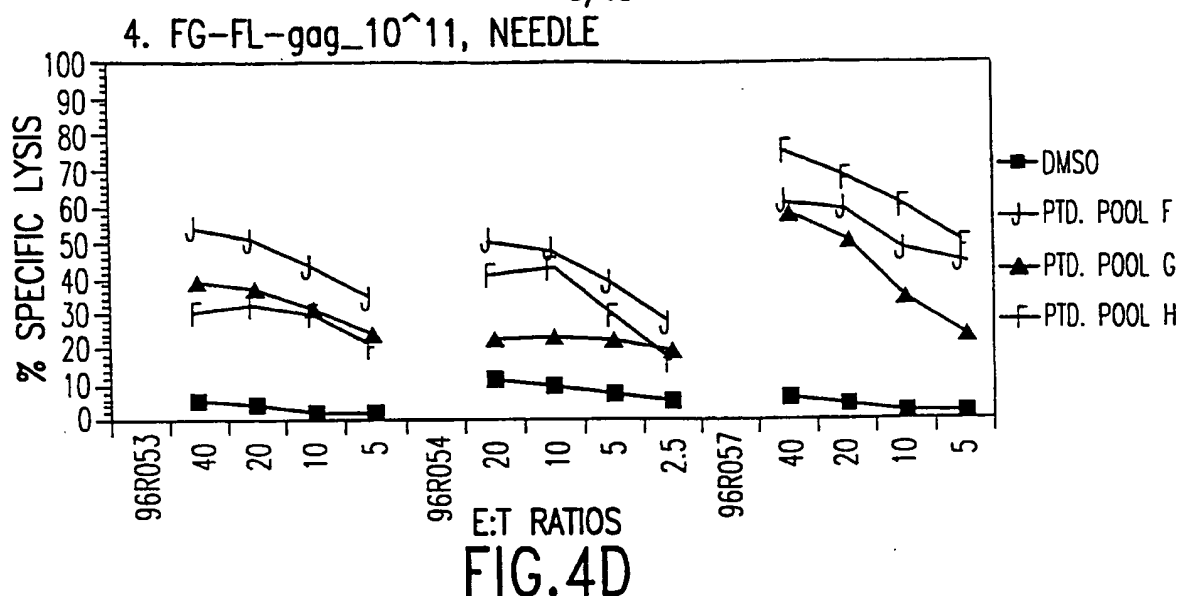
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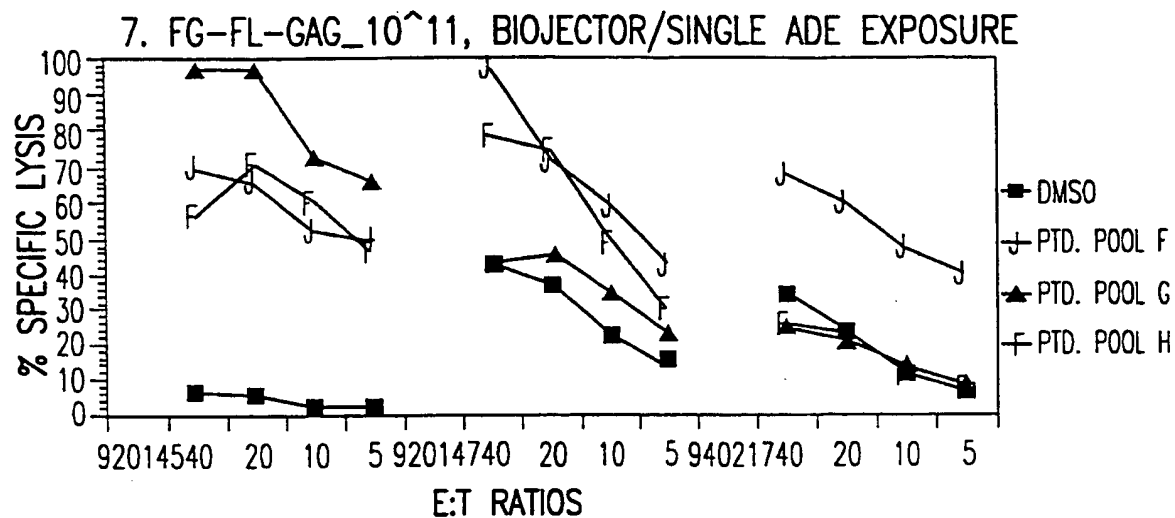


FIG.4G

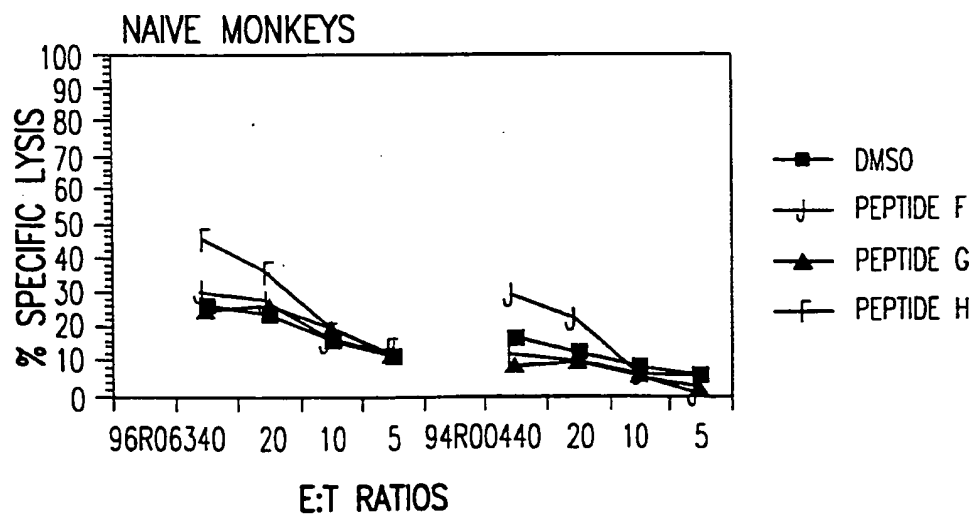


FIG.4H

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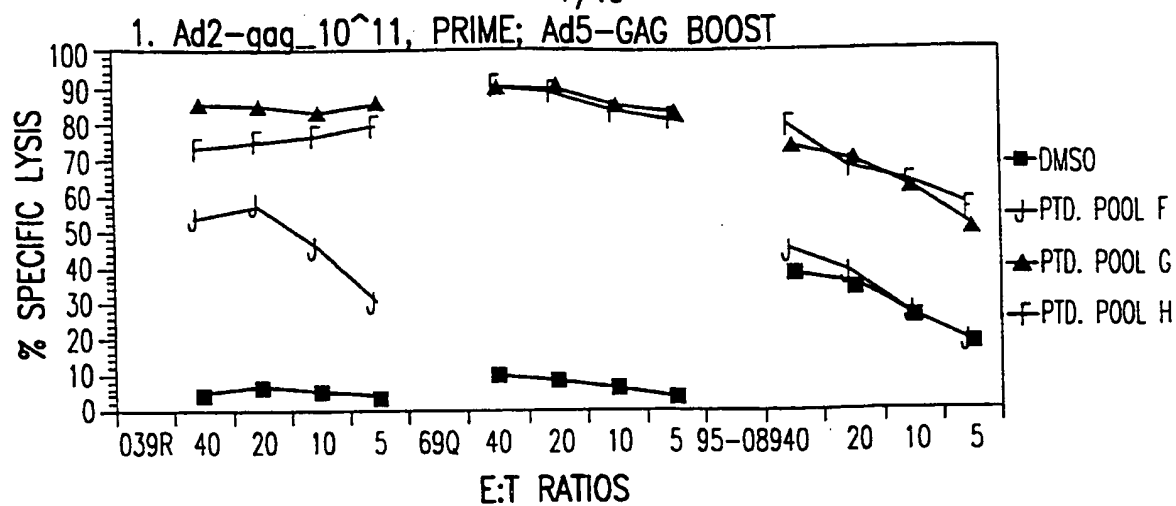


FIG.5A

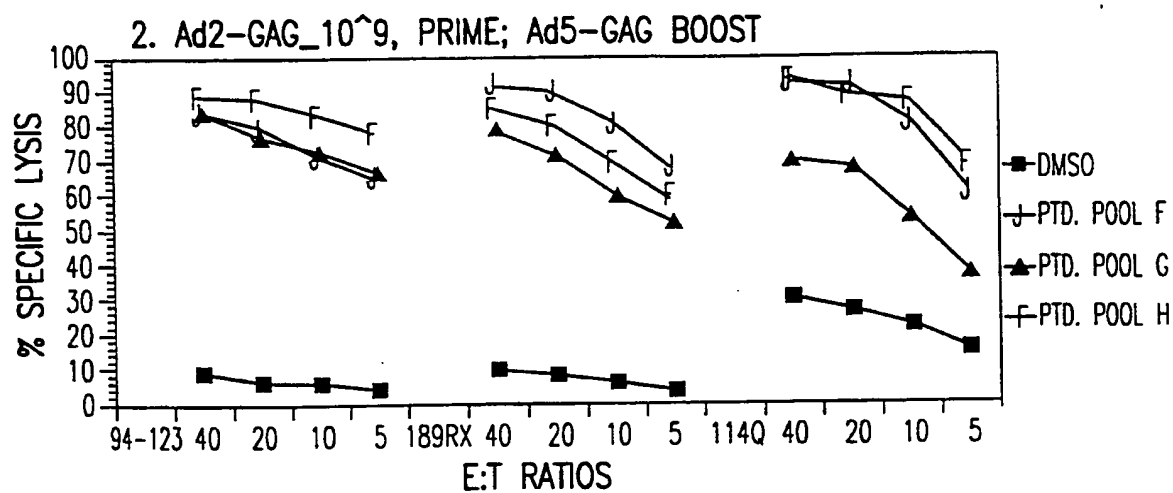


FIG.5B

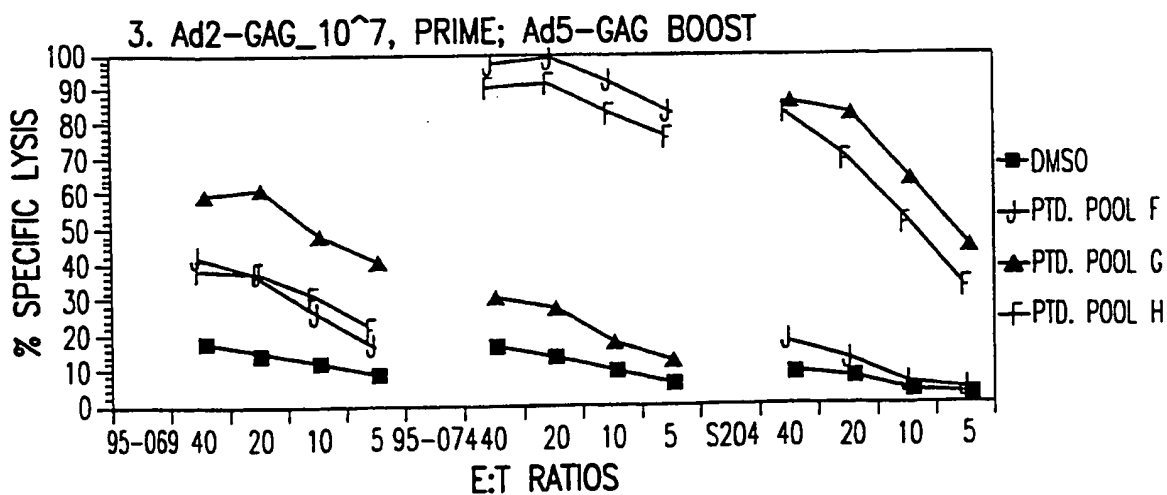


FIG.5C

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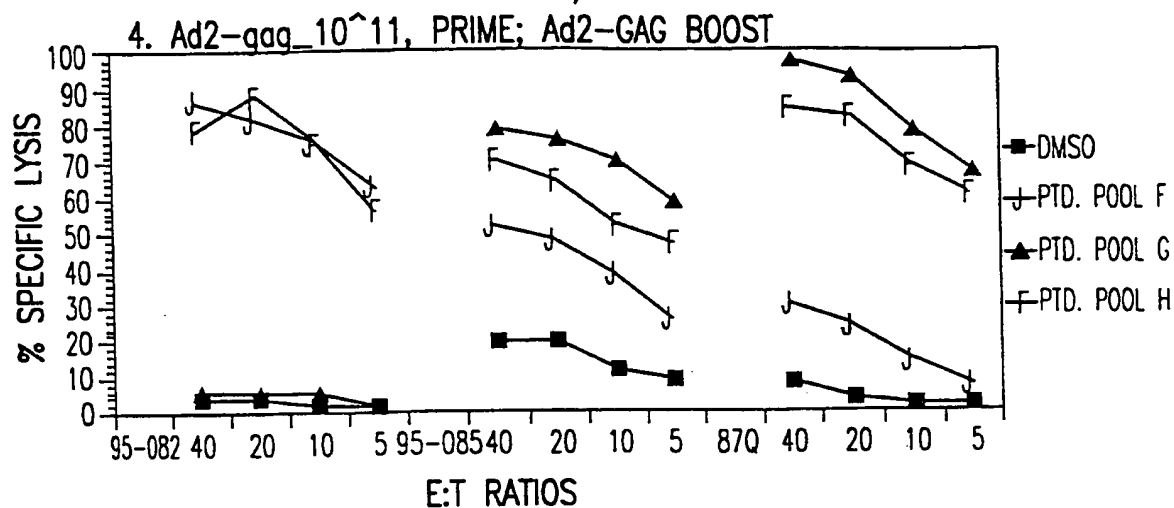


FIG.5D

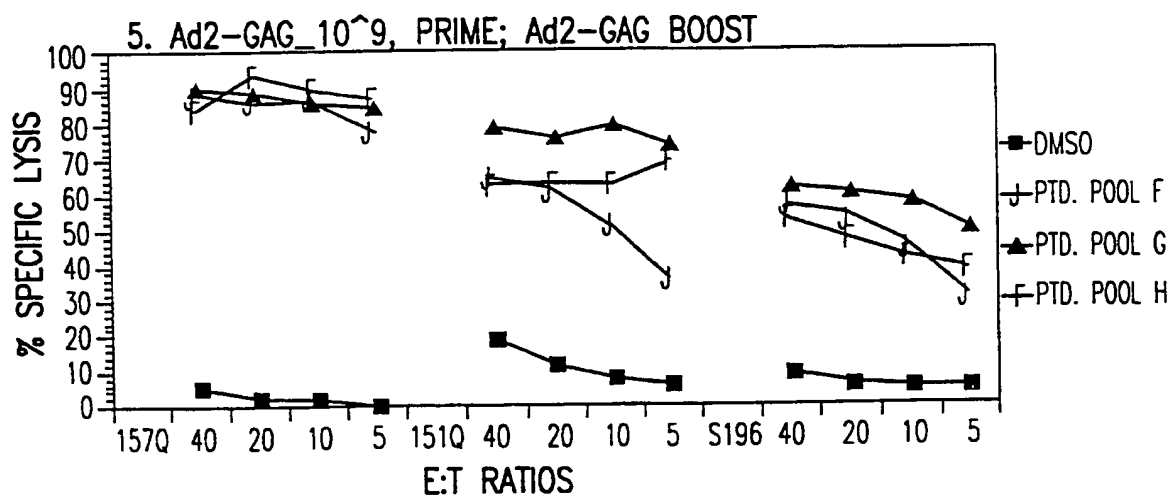


FIG.5E

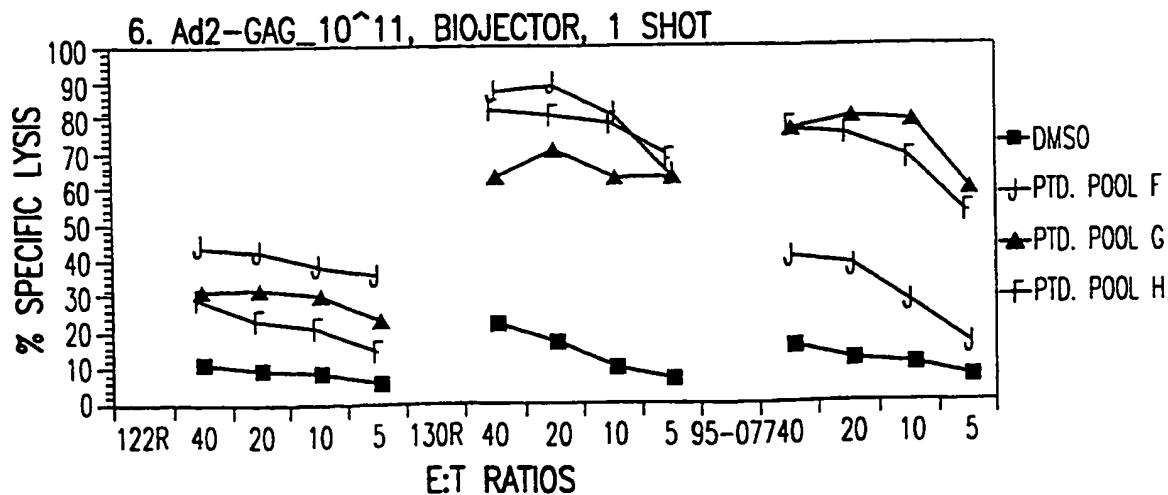
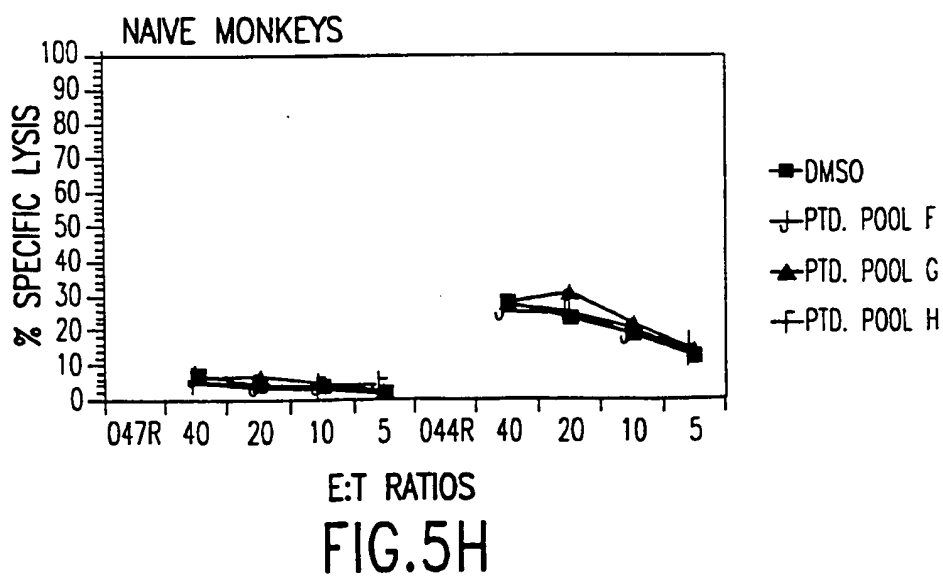
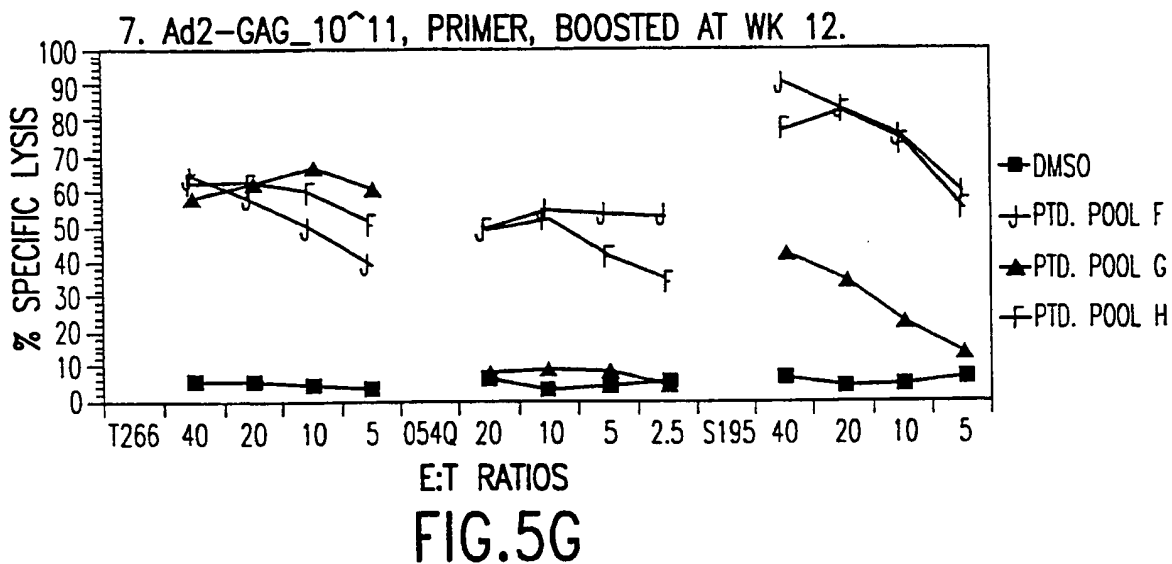


FIG.5F

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Optimized HIV-1 (CAM1) *gag* orf

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51 CAAGTGGGAG AAGATCAGGC TGAGGCCTGG TGGCAAGAAG AAGTACAAGC
101 TAAAGCACAT TGTGTGGGCC TCCAGGGAGC TGGAGAGGTT TGCTGTGAAC
151 CCTGGCCTGC TGGAGACCTC TGAGGGGTGC AGGCAGATCC TGGGCCAGCT
201 CCAGCCCTCC CTGCAAACAG GCTCTGAGGA GCTGAGGTCC CTGTACAACA
251 CAGTGGCTAC CCTGTACTGT GTGCACCAGA AGATTGATGT GAAGGACACC
301 AAGGAGGCCC TGGAGAAGAT TGAGGAGGAG CAGAACAAGT CCAAGAAGAA
351 GGCCAGCAG GCTGCTGCTG GCACAGGCAA CTCCAGCCAG GTGTCCCAGA
401 ACTACCCCAT TGTGCAGAAC CTCCAGGGCC AGATGGTGCA CCAGGCCATC
451 TCCCCCGGA CCCTGAATGC CTGGGTGAAG GTGGTGGAGG AGAAGGCCTT
501 CTCCCCTGAG GTGATCCCCA TGTCTCTGC CCTGTCTGAG GGTGCCACCC
551 CCCAGGACCT GAACACCATG CTGAACACAG TGGGGGGCCA TCAGGCTGCC
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FIG.6

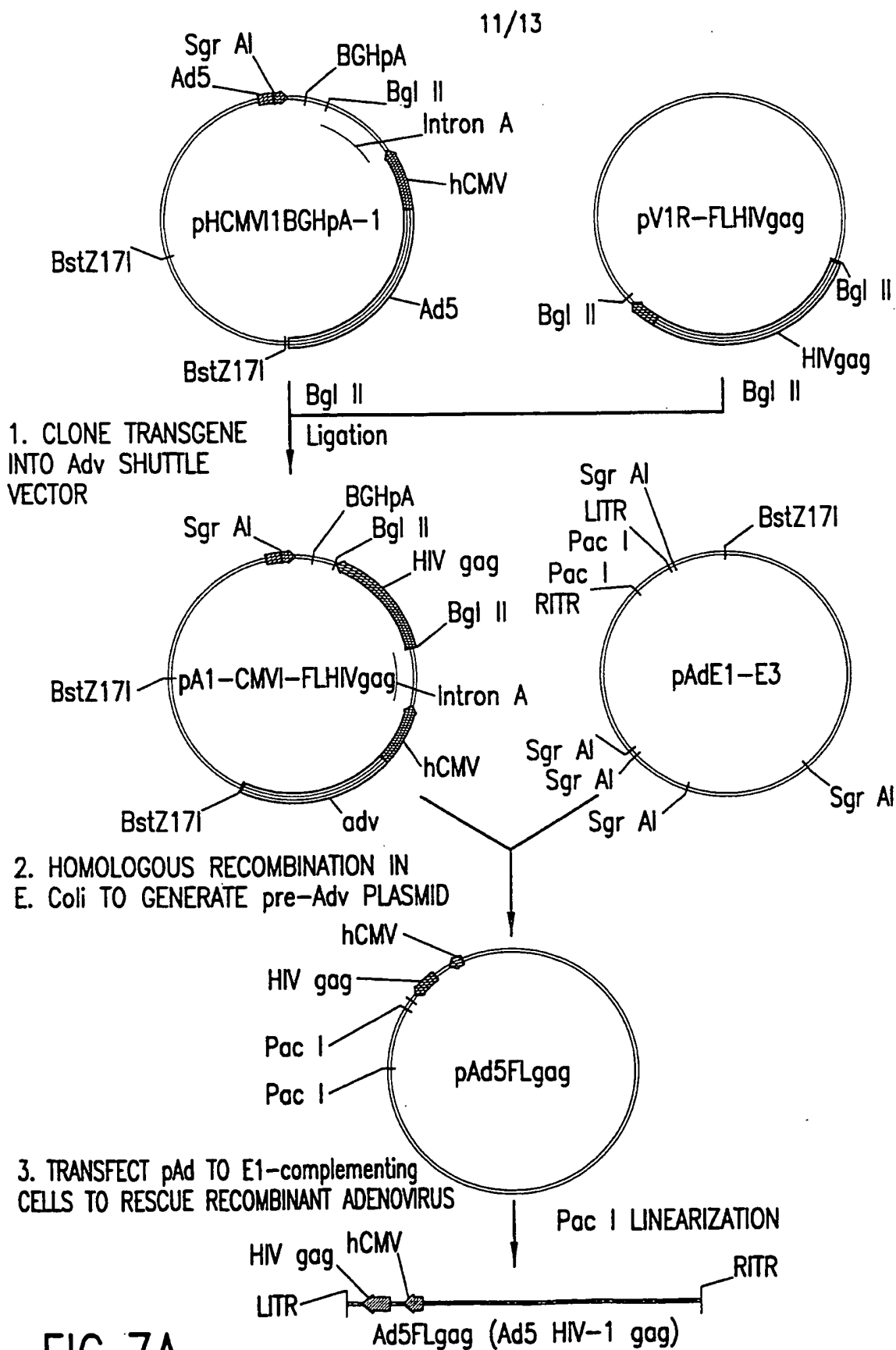


FIG. 7A

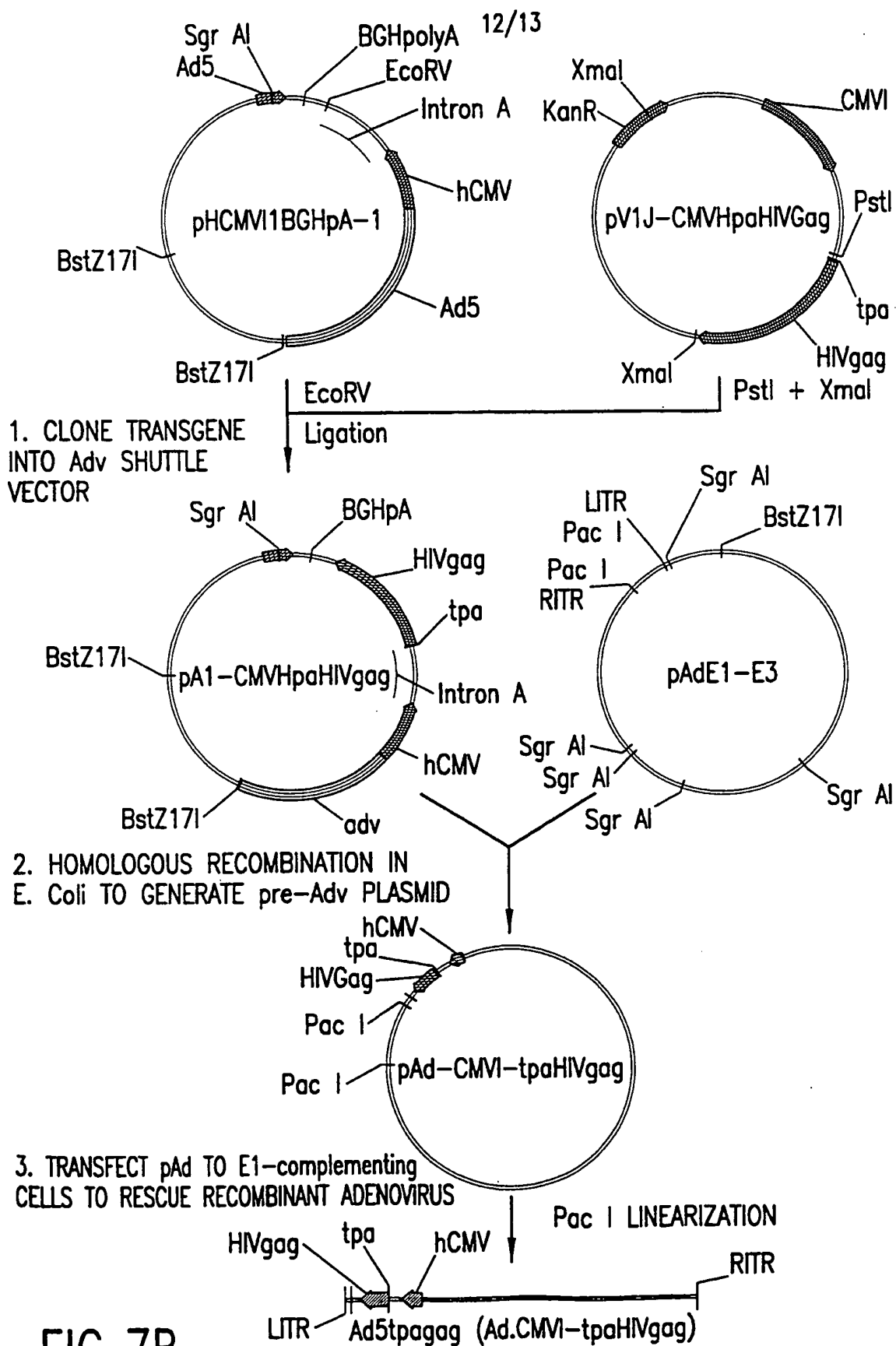


FIG. 7B

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TPA-GAG open reading frame

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FIG. 8

SEQUENCE LISTING

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<150> 60/142,631

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Minimum documentation searched (classification system followed by classification symbols)

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPT, JPAB, EPAB, DWPI; defective adenoviral vector, hiv gag, codon optimized, heterologous promoter, vaccine,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,859,193 A (DEVARE et al) 12 January 1999, column 3, lines 26-37, columns 5-7, Example 1.	1-20
Y	US 5,672,508 A (GYURIS et al) 30 September 1997, column 17, lines 14-56.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

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(54) Title: PRIME-BOOST VACCINATION STRATEGY

(57) Abstract: The present invention provides a method for inducing an immune response to an antigen in a subject. The method comprises administering to the subject DNA encoding the antigen, and subsequently orally administering to the subject a composition comprising transgenic material, wherein the transgenic material comprises a DNA molecule encoding the antigen such that the antigen is expressed in the transgenic material.

PRIME-BOOST VACCINATION STRATEGY

FIELD OF THE INVENTION:

The present invention relates to a method for inducing an immune
5 response to an antigen in a subject.

BACKGROUND OF THE INVENTION:

Measles is a highly contagious viral disease that has persisted for more
than 1000 years since it was first described (Babbott and Gordon, 1954).
10 Severe infection may lead to pneumonia, encephalitis (brain inflammation)
and death. Although measles can be effectively prevented by a
live-attenuated vaccine (LAV) it still causes approximately 800,000 deaths
every year, predominantly among children in developing countries (Cutts
and Steinglass, 1998).

15 The inability to control measles using the LAV is largely due to
neutralization of the vaccine by maternal antibodies. In order to avoid
neutralization by maternal antibodies the LAV is generally administered
between 12 and 18 months. However maternal antibodies may decline more
rapidly in infants of developing countries (Gans *et al.*, 1998). As a
20 consequence, there is a window between 6 and 18 months of age during
which infants may lack both passive and active immunity.

An additional concern is the effective distribution and use of live
attenuated measles vaccines in developing countries in particular the
maintenance of the "cold chain" during transport and storage to ensure the
25 viability of the vaccine prior to administration. This, together with
requirement for trained staff for parenteral application of the vaccine, has led
to poor vaccination coverage in these countries.

In an attempt to overcome the problem of maternal antibodies a high
titre Edmonston-Zagreb vaccine was given to young infants in the late 1980's.
30 This vaccine protected infants against measles but led to an increased
mortality from other infections such as diarrhoea and pneumonia (Markowitz
et al., 1990; Garenne *et al.*, 1991) and was subsequently withdrawn from use
in 1992 (Weiss, 1992). It is thought that the increase in mortality was due to
an immunosuppressive effect similar to that seen with wild type infection.

35 Sub-unit vaccines are not subject to the same constraints as LAVs.
Development of a sub-unit vaccine for measles would primarily address

issues concerning the immunization and protection of children in the developing world, such as maternal antibodies. In addition to this non-replicating sub-unit vaccines cannot initiate infection in immuno-compromised patients. New vaccine approaches such as DNA subunit vaccines and edible subunit vaccines are currently being devised as alternatives to the LAV. The measles virus (MV) hemagglutinin (H) protein is an immunodominant surface exposed glycoprotein and has been incorporated into these vaccines.

A number of studies have been conducted using DNA vaccines encoding the MV-H protein. The immune responses generated have been of varying success. Cardoso *et al.* (1996) demonstrated that intramuscular inoculation of BALB/c mice with a secreted form of plasmid DNA encoding the H protein induced a class I-restricted CTL response and IgG1 antibody production (consistent with a T_H2 -type response). Furthermore, antibody responses were not increased by multiple inoculations. In contrast, Yang *et al.* (1997) found that neutralizing antibody titres increased 2- to 4-fold in BALB/c mice following repeated gene-gun inoculations. In addition, these titres were better than those raised by the LAV. When similar plasmid constructs were used for macaque vaccination, however, antibody levels were found to be 100-fold lower than those elicited by a single dose of the LAV (Polack *et al.*, 2000). Such studies highlight the dependence of an appropriate immune response on the number and route of administrations used in each particular animal model.

Bacterial and viral antigens have been expressed in transgenic plants and transiently from plant viral vectors. Antigens from both sources retain their native immunogenic properties and are able to induce neutralizing and protective antibodies in mice (Haq *et al.*, 1995; Mason *et al.*, 1996; Arakawa *et al.*, 1998; Tacket *et al.*, 1998; Wigdorovitz *et al.*, 1999A & B). Systemic and mucosal immune responses have also been induced in human volunteers feed raw potato tubers expressing the binding subunit of the *E. coli* heat labile enterotoxin (LT-B) (Tacket *et al.* 1998). The serum antibodies produced by these volunteers were able to neutralize *E. coli* heat labile enterotoxin (LT) *in vitro*. Thus, the current data demonstrates that oral vaccination with plant-derived antigens can evoke a protective immune response.

The present invention provides an alternate strategy for inducing an immune response to an antigen in a subject. Also provided are transgenic plants expressing an antigen derived from the measles virus.

5 **SUMMARY OF THE INVENTION:**

In a first aspect, the present invention provides a method for inducing an immune response to an antigen in a subject, the method comprising administering to the subject DNA encoding the antigen, and subsequently orally administering to the subject a composition comprising transgenic
10 material, wherein the transgenic material comprises a DNA molecule encoding the antigen such that the antigen is expressed in the transgenic material.

In a preferred embodiment of the present invention the composition further comprises a mucosal adjuvant, preferably cholera toxin β -subunits.

15 It is also preferred that the antigen is expressed in the transgenic material as a fusion protein. In particular it is preferred the fusion protein comprises the antigen C-terminally fused to the amino acid sequence SEKDEL (SEQ ID NO:1).

The transgenic material is preferably a transgenic plant such as a fruit
20 or vegetable. It is preferred that the transgenic plant is selected from the group consisting of; tobacco, lettuce, rice and bananas.

In a further preferred embodiment of the present invention, the antigen is selected from the group consisting of viral antigens, parasitic antigens and bacterial antigens, preferably measles virus, the human
25 immunodeficiency virus, or Plasmodium sp. It is preferred that the antigen is the measles virus H or F protein, or fragments thereof, preferably the measles H protein.

In a still further preferred embodiment the DNA encoding the antigen is administered to the subject on at least two occasions and the composition
30 comprising transgenic material is orally administered to the subject on at least two occasions. More preferably, the DNA encoding the antigen is administered to the subject on a single occasion and the composition comprising transgenic material is orally administered to the subject on a single occasion.

35 In a second aspect the present invention provides a transgenic plant, the plant having been transformed with a DNA molecule, the DNA molecule

comprising a sequence encoding a measles virus antigen such that the plant expresses the measles virus antigen.

In a preferred embodiment of this aspect of the invention, the DNA molecule encodes a fusion protein, preferably comprising the measles
5 antigen C-terminally fused to the amino acid sequence SEKDEL.

In a further preferred embodiment the measles antigen is the measles H protein.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a
10 stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The invention will hereinafter be described by way of the following non-limiting Figures and Examples.

15

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS:

Figure 1: Plant transformation vector constructs for expression of MV-H protein in tobacco. The T-DNA region inserted into the plant genome contains the nopaline synthase expression cassette (Kan^R), which confers
20 kanamycin resistance on transformed cells, and the MV-H protein expression cassette. The MV-H protein expression cassette comprises a cauliflower mosaic virus 35S promoter (35S-Pro) fused to a tobacco etch virus 5'-untranslated region (TEV) and cauliflower mosaic virus terminator sequence (35S-Ter). The pBinH/KDEL and pBinSP/H/KDEL constructs contain an
25 SEKDEL peptide sequence (KDEL) fused to the C-terminal end of the H protein for retention in the endoplasmic reticulum. The pBinSP/H/KDEL construct also contains a plant signal peptide (SP) fused to the N-terminal end of the H protein.

30 Figure 2: Transgene expression and production of recombinant MV-H protein in transgenic tobacco. (A) Northern blot comparing the level of MV-H gene expression of the six highest expressing T₀ transgenic tobacco lines obtained for each MV-H construct. Each lane contained 10 µg of total RNA and was probed with a ³²P-labeled MV-H cDNA probe. (B) ELISA analysis of
35 MV-H protein expression in each of the T₀ transgenic tobacco lines shown in (A) detected with a rabbit anti-measles polyclonal antibody. Four

independent control transgenic lines transformed with a pBin construct lacking the MV-H gene, were included in analyses.

Figure 3: Detection of MV-H protein in pBinH/KDEL T₁ transgenic lines.

- 5 Selected kanamycin resistant progeny from the three highest T₀ expressing lines (8B, 12C and 39H) were analysed for MV-H protein expression using ELISA. The analysis was performed using either a rabbit anti-measles polyclonal antibody or MV-positive human serum. Control extract is from a transgenic tobacco line transformed with a pBin construct lacking the MV-H gene.
- 10

Figure 4: Immune response in mice following intraperitoneal (IP)

- immunization with transgenic plant extracts. Five mice were immunized with leaf extract from pBinH/KDEL T₁ transgenic line 8B or a pBin control transgenic line. IP immunizations were delivered on days 0, 14 and 49 with serum collected on days 28 and 84. (A) MV-specific serum IgG. Control serum is the mean value obtained from 3- 4 naïve mice. (B) MV neutralization activity of serum IgG from day 84. MV-H (●), control (○).
- 15

Figure 5: Immune response in mice following gavage with transgenic plant extracts. (A) Mouse serum neutralization titres following gavage. Sera collected 49 days after initial treatment were pooled and the neutralizing ability against MV assessed in plaque-reduction neutralization (PRN) assays. Naïve (◆), 2g MV-H + CT-CTB (▲), and 2g control + CT-CTB (■).

- (B) MV-specific secretory IgA in faecal isolates collected 28 days after initial gavage.
- 20
- 25

Figure 6: Serum MV neutralization (PRN) titres following DNA vaccination of mice. Sera collected 0, 15, 43 and 140 days after DNA vaccination were pooled. Naïve (◆), 2g MV-H + CT-CTB (▲), and 2g control + CT-CTB (■).

30

Figure 7: MV-specific serum IgG titres following DNA-oral prime boost vaccination. Serum IgG titres were determined by ELISA on pooled sera from 0, 21 (pre-boost) and 49 days (post-boost). (A) MV-specific serum IgG titres for mice immunized with MV-H DNA and boosted with MV-H (-▲-), or control (-■-) plant extracts. (B) MV-specific serum IgG titres for mice

35

immunized with control DNA and boosted with MV-H (-▲-), or control (-■-) plant extracts. (C) Actual IgG titres represented in A and B.

Figure 8: Serum MV neutralization (PRN) titres following DNA-oral prime
5 boost vaccination of mice. Neutralization titres were determined using
pooled sera from 0, 21 (pre-boost) and 49 days (post-boost). (A)
Neutralization titre for mice immunized with MV-H DNA and boosted with
MV-H (-▲-), or control (-■-) plant extracts. (B) Neutralization titre for mice
immunized with control DNA and boosted with MV-H (-▲-), or control (-■-)
10 plant extracts. (C) Actual neutralization titres represented in A and B.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS:

Unless otherwise indicated, the recombinant DNA techniques utilized
in the present invention are standard procedures, well known to those skilled
15 in the art. Such techniques are described and explained throughout the
literature in sources such as, J. Perbal, A Practical Guide to Molecular
Cloning, John Wiley and Sons (1984); J. Sambrook et al., Molecular Cloning:
A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989); T.A.
Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes
20 1 and 2, IRL Press (1991); D.M. Glover and B.D. Hames (editors), DNA
Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996); and
F.M. Ausubel et al. (editors), Current Protocols in Molecular Biology, Greene
Pub. Associates and Wiley-Interscience (1988, including all updates until
present) and are incorporated herein by reference.

25 DNA vaccination involves the direct *in vivo* introduction of DNA
encoding an antigen into tissues of a subject for expression of the antigen by
the cells of the subject's tissue. Such vaccines are termed herein "DNA
vaccines" or "nucleic acid-based vaccines." DNA vaccines are described in
US 5,939,400, US 6,110,898, WO 95/20660 and WO 93/19183, the
30 disclosures of which are hereby incorporated by reference in their entireties.
The ability of directly injected DNA that encodes an antigen to elicit a
protective immune response has been demonstrated in numerous
experimental systems (see, for example, Conry et al., 1994; Cardoso *et al.*,
1996; Cox et al., 1993; Davis et al., 1993; Sedegah et al., 1994; Montgomery et
35 al., 1993; Ulmer et al., 1993; Wang et al., 1993; Xiang et al., 1994; Yang et al.,
1997).

To date, most DNA vaccines in mammalian systems have relied upon viral promoters derived from cytomegalovirus (CMV). These have had good efficiency in both muscle and skin inoculation in a number of mammalian species. A factor known to affect the immune response elicited by DNA immunization is the method of DNA delivery, for example, parenteral routes can yield low rates of gene transfer and produce considerable variability of gene expression (Montgomery et al., 1993). High-velocity inoculation of plasmids, using a gene-gun, enhanced the immune responses of mice (Fynan et al., 1993; Eisenbraun et al., 1993), presumably because of a greater efficiency of DNA transfection and more effective antigen presentation by dendritic cells. Vectors containing the nucleic acid-based vaccine of the invention may also be introduced into the desired host by other methods known in the art, e.g., transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), or a DNA vector transporter.

"Transgenic material" of the present invention refers to any substance of biological origin that has been genetically engineered such that it produces the antigen. Preferably, the transgenic material is a transgenic plant.

The orally administered composition can be administered by the consumption of a foodstuff, where the edible part of the transgenic material is used as a dietary component while the antigen is provided to the subject in the process.

The present invention allows for the production of not only a single antigen in the DNA vaccine and/or the transgenic material but also allows for a plurality of antigens.

DNA sequences of multiple antigenic proteins can be included in the expression vector used for transformation of an organism, thereby causing the expression of multiple antigenic amino acid sequences in one transgenic organism. Alternatively, an organism may be sequentially or simultaneously transformed with a series of expression vectors, each of which contains DNA segments encoding one or more antigenic proteins. For example, there are five or six different types of influenza, each requiring a different vaccine. Transgenic material expressing multiple antigenic protein sequences can simultaneously boost an immune response to more than one of these strains, thereby giving disease immunity even though the most prevalent strain is not known in advance.

Plants which are preferably used in the practice of the present invention include any dicotyledon and monocotyledon which is edible in part or in whole by a human or an animal such as, but not limited to, carrot, potato, apple, soybean, rice, corn, berries such as strawberries and
5 raspberries, banana and other such edible varieties. It is particularly advantageous in certain disease prevention for human infants to produce a vaccine in a juice for ease of oral administration to humans such as tomato juice, soy bean milk, carrot juice, or a juice made from a variety of berry types. Other foodstuffs for easy consumption include dried fruit.

10 Several techniques exist for introducing foreign genetic material into a plant cell, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include acceleration of genetic material coated onto microparticles directly into cells (see, for example, US 4,945,050 and US 5,141,131). Plants may be transformed using Agrobacterium
15 technology (see, for example, US 5,177,010, US 5,104,310, US 5,004,863, US 5,159,135). Electroporation technology has also been used to transform plants (see, for example, WO 87/06614, US 5,472,869, 5,384,253, WO 92/09696 and WO 93/21335). Each of these references are incorporated herein by reference. In addition to numerous technologies for transforming
20 plants, the type of tissue which is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue type I and II, hypocotyl, meristem, and the like. Almost all plant tissues may be transformed during development and/or differentiation using appropriate techniques described herein.

25 A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels et al., Cloning Vectors: A Laboratory Manual, 1985, supp. 1987; Weissbach and Weissbach, Methods for Plant Molecular Biology, Academic Press, 1989; and Gelvin et al., Plant Molecular Biology Manual, Kluwer Academic
30 Publishers, 1990. Typically, plant expression vectors include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant expression vectors also can contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or
35 developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an

RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

Examples of plant promoters include, but are not limited to ribulose-1,6-bisphosphate carboxylase small subunit, beta-conglycinin promoter, phaseolin promoter, ADH promoter, heat-shock promoters and tissue specific promoters. Promoters may also contain certain enhancer sequence elements that may improve the transcription efficiency. Typical enhancers include but are not limited to Adh-intron 1 and Adh-intron 6.

Constitutive promoters direct continuous gene expression in all cells types and at all times (e.g., actin, ubiquitin, CaMV 35S). Tissue specific promoters are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (e.g., zein, oleosin, napin, ACP, globulin and the like) and these promoters may also be used. Promoters may also be active during a certain stage of the plants' development as well as active in plant tissues and organs. Examples of such promoters include but are not limited to pollen-specific, embryo specific, corn silk specific, cotton fiber specific, root specific, seed endosperm specific promoters and the like.

Under certain circumstances it may be desirable to use an inducible promoter. An inducible promoter is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes); light (RUBP carboxylase); hormone (Em); metabolites; and stress. Other desirable transcription and translation elements that function in plants may be used.

In addition to plant promoters, promoters from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoters of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S) and the like may be used.

A number of plant-derived edible vaccines are currently being developed for both animal and human pathogens (Hood and Jilka, 1999). Immune responses have also resulted from oral immunization with transgenic plants producing virus-like particles (VLPs), or chimeric plant viruses displaying antigenic epitopes (Mason *et al.*, 1996; Modelska *et al.*, 1998; Kapustra *et al.*, 1999; Brennan *et al.*, 1999). It has been suggested that the particulate form of these VLPs or chimeric viruses may result in greater

stability of the antigen in the stomach, effectively increasing the amount of antigen available for uptake in the gut (Mason *et al.* 1996, Modelska *et al.* 1998).

5 Mutant and variant forms of the DNA sequences encoding for a particular antigen may also be utilized in this invention. For example, expression vectors may contain DNA coding sequences which are altered so as to change one or more amino acid residues in the antigen expressed in the transgenic material, thereby altering the antigenicity of the expressed protein. Expression vectors containing a DNA sequence encoding only a
10 portion of an antigenic protein as either a smaller peptide or as a component of a new chimeric fusion protein are also included in this invention.

The present invention can be used to produce an immune response in animals other than humans. Diseases such as: canine distemper, rabies, canine hepatitis, parvovirus, and feline leukemia may be controlled with
15 proper immunization of pets. Viral vaccines for diseases such as: Newcastle, Rinderpest, hog cholera, blue tongue and foot-mouth can control disease outbreaks in production animal populations, thereby avoiding large economic losses from disease deaths. Prevention of bacterial diseases in production animals such as: brucellosis, fowl cholera, anthrax and black leg
20 through the use of vaccines has existed for many years. The transgenic material used in the methods of the present invention may be incorporated into the feed of animals.

A "mucosal adjuvant" is a compound which non-specifically stimulates or enhances a mucosal immune response (e.g., production of IgA antibodies).
25 Administration of a mucosal adjuvant in a composition facilitates the induction of a mucosal immune response to the immunogenic compound.

The mucosal adjuvant may be any mucosal adjuvant known in the art which is appropriate for human or animal use. For example, the mucosal adjuvant may be cholera toxin (CT), enterotoxigenic *E. Coli* heat-labile toxin (LT), or a derivative, subunit, or fragment of CT or LT which retains
30 adjuvanticity. Preferably, the mucosal adjuvant is cholera toxin β -subunits. The mucosal adjuvant is co-administered with the composition comprising transgenic material in an amount effective to elicit or enhance a mucosal immune response. The suitable amount of adjuvant may be determined by
35 standard methods by one skilled in the art. Preferably, the adjuvant is

present at a ratio of 1 part adjuvant to 10 parts composition comprising the transgenic material.

In the present invention, the antigen can be expressed in the transgenic material as a fusion protein. Typically, the additional amino acid sequence will extend from the C-terminus and/or the N-terminus of the antigen. Preferably, the fusion protein results in a higher immune response when compared to when the antigen not expressed as a fusion protein. It is also preferred that the fusion protein comprise at least two antigens from the same or different native protein. In the latter instance, the different antigens can be from different organisms, providing immune protection against a number of pathogens.

Example

Experimental Protocol

15 *Construction of transgenic tobacco plants producing H protein*

Three constructs were generated for the expression of MV-H protein in tobacco plants (Figure 1) (a) pBinH – H protein alone, (b) pBinH/KDEL – addition of a C-terminal endoplasmic reticulum (ER)-retention sequence and (c) pBinSP/H/KDEL – addition of both an N-terminal plant signal peptide and a C-terminal ER-retention sequence.

To produce these constructs a 1.8 kb *EcoRI* / *BamHI* fragment encompassing the open reading frame of the MV-H gene (Edmonston strain; GenBank accession no. X16565) was obtained from plasmid pBS-HA (Johns Hopkins Hospital, Baltimore). Using the Altered Sites kit (Promega) an *NcoI* site was introduced into the 5'-end of the H gene. The *NcoI* site was created around the existing initiation codon by mutating the first nucleotide of the second codon from T to C. This also altered the second amino acid of the H protein from serine to alanine. The *NcoI* / *BamHI* fragment containing the N-terminal modified H gene was then transferred into the plant expression vector pRTL2 (Restrepo *et al.*, 1990) to give pRTL2-H.

A second H-protein construct containing the *NcoI* site described above and an endoplasmic reticulum-retention sequence SEKDEL (Munro and Pelham, 1987) was also engineered. A *XhoI* site was introduced into the C-terminus of the H gene immediately upstream of the stop codon and *BamHI* site using the Altered Sites kit (Promega). This allowed a double-stranded oligonucleotide encoding the SEKDEL sequence to be ligated between the

*Xho*I and *Bam*HI sites creating an in-frame fusion with the C-terminal end of the H protein. The SEKDEL oligonucleotide was produced by annealing the following complementary sequences: 5'-

- TCGATCTCTGAGAAAGATGAGCTATGAGGG-3' (SEQ ID NO:2) and 5'-
 5 GATCCCCTCATAGCTCAT CTTTCTCAGAGA-3' (SEQ ID NO:3). The C-terminal sequence of the modified H protein was altered from TNRR* (SEQ ID NO:4) to TNLQSEKDEL* (SEQ ID NO: 5). The H/KDEL fragment was then cloned into pRTL2 to give pRTL2-H/KDEL.

- In the third construct, the signal peptide (SP) of the tobacco *Pr1a* gene
 10 (Hammond-Kosack *et al.* 1994) was cloned into the *Nco*I site of pRTL2-H/KDEL upstream of, and in frame with, the H protein. The 107 bp SP fragment was amplified by PCR from the plasmid SLJ6069 (Sainsbury Laboratory, JIC, Norwich, UK) using the oligonucleotides: 5'-
 GCGCCATGGGATTTGTTCTCTTT-3' (SEQ ID NO: 6) and 5'-
 15 TATCCATGGGCCCGGCACGGCAAGAGTGGGATAT-3' (SEQ ID NO:7). This clone was designated pRTL2-SP/H/KDEL.

- Following verification of modifications by sequence analysis, the expression cassettes of pRTL2-H, pRTL2-H/KDEL, and pRTL2-SP/H/KDEL were transferred into the binary vector pBin19 (Bevan, 1984) to produce
 20 pBinH, pBinH/KDEL and pBinSP/H/KDEL, respectively (Figure 1).

These three constructs were then electroporated into *Agrobacterium tumefaciens* strain LBA 4404 and used for transformation of tobacco (*Nicotiana tabacum* var Samsun) using the leaf disc method as described by Horsch *et al.* (1985).

25

Transgene expression analysis

- Total RNA was extracted from 150mg leaf samples of *in vitro* transgenic tobacco plants in 0.1M Tris, 0.1M NaCl, 10 mM EDTA, 1% SDS, 1% β -mercaptoethanol, pH 9.0 by extracting twice with an equal volume of
 30 phenol and once with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v). The final aqueous phase was mixed with 0.1 volume of sodium acetate (pH 5.0) and 2.5 volumes of cold 100% ethanol, incubated at -20°C for 30 min and nucleic acid pelleted by centrifugation at 13,000 g for 10 min. The pellet was rinsed with cold 70% ethanol, dried and resuspended in 25 μ l
 35 of sterile water. RNA was analysed by northern blot using a ³²P-labelled MV-H cDNA probe.

Detection of MV-H protein in transgenic tobacco by ELISA

Tobacco leaves (50mg) were frozen in liquid nitrogen and ground to a fine powder in a 1.5 ml eppendorf. Five volumes of chilled extraction buffer
5 (PBS containing 100mM ascorbic acid, 20mM EDTA, 0.1% Tween-20 and 1mM PMSF, pH 7.4) was added and the extract vortexed for 15 s. The extract was then centrifuged at 23,000 g for 15 min at 4°C, the supernatant collected and glycerol added to a final concentration of 16% before snap freezing in liquid nitrogen and storage at -70°C.

10 Plant extracts were diluted in 0.1M carbonate buffer (pH 9.6) and were coated onto ELISA plates at 4°C overnight. All further incubations were at 37°C for 1 hour. Following a blocking step with 2.5% skim milk the MV-H protein was detected with a rabbit polyclonal anti-measles antibody (CDC, Atlanta) diluted 1/4000. Anti-rabbit horseradish peroxidase conjugate
15 (Boehringer Mannheim) diluted 1/8000 was used as the secondary antibody. The plates were developed with TMB (3,3',5,5'-tetramethylbenzidine) substrate for 30 - 60 min and read at 630nm.

Preparation of antigen from transgenic plants

20 Recently expanded leaves from glasshouse grown plants of the pBinH/KDEL transgenic line 8B, or transgenic tobacco lacking the MV-H gene, were harvested and stored at -35°C. All subsequent steps were performed on ice or at 4°C. Frozen tobacco leaves were powdered in a coffee grinder and mixed with 2.5 volumes of chilled extraction buffer (described
25 above). The extract was filtered through 2 layers of miracloth, centrifuged at 100g for 5 min and the supernatant centrifuged again at 32,600 g for 60 min. Glycerol was added to the pellet to a final concentration of 16% allowing the extracts to be stored at -70°C. Extracts ranged in concentration from 3.2g/ml to 4.5g/ml.

30 The supernatant from the 32,600g spin was further purified. Proteins precipitated from the supernatant between 25% and 50% ammonium sulphate (AS) were resuspended in phosphate buffered saline (PBS) containing 10 mM ascorbic acid, and applied to PD-10 columns (Amersham Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated with PBS. The
35 protein fraction was eluted in PBS, glycerol was added to a final concentration of 16% allowing the extracts to be stored at -70°C.

A mucosal adjuvant consisting of 2µg of cholera toxin (CT) and 10µg of cholera toxin B subunit (CTB) (Sigma, USA) was added to plant aliquots immediately prior to gavage. Gavage was performed using an 8cm gavage needle attached to a 1ml Tuberculin syringe. The gavage needle was inserted
5 down the oesophagus of anaesthetized animals into the stomach, where 0.4g, 1g, 2g or 4g of plant material was injected. Mice were studied for signs of tracheal or nasal obstruction until fully recovered from anaesthetic.

Laboratory mice and cell lines

10 Adult female Balb/c mice, between 18-25g (approximately 8 weeks old), were purchased from Animal Research Centre, Western Australia, and were maintained in the University Animal House. Rhesus monkey kidney cells (RMK cells) were grown as monolayers at 37°C in RPMI 1640 medium (Trace, Biosciences Ltd, Australia) supplemented with 10% fetal calf serum
15 (FCS) (Trace) in a 5% CO₂ atmosphere.

Construction and vaccination of MV-H DNA

A high copy pCI plasmid vector (Promega, USA) incorporating a human cytomegalovirus (CMV) immediate-late enhancer/promoter,
20 ampicillin resistance and the SV40 late polyadenylation signal was used for vaccine production. Two DNA vaccine constructs were prepared. One containing the extracellular domain of the measles virus H gene (MV-H), and a control construct containing the ovalbumin gene.

A 1ml Insulin needle (Becton Dickinson, USA) was used to inject 25 or
25 50µg of DNA solution into both quadriceps of each mouse.

Collection of mouse samples

Blood was collected by intraocular bleeding or cardiac puncture, once blood had clotted serum was recovered by centrifugation (7100g, 6 min).

30 Faeces were collected into eppendorfs pre-blocked with 1% BSA. 1ml of 0.1% BSA + 0.15mM PMSF solution in PBS was added per 100mg of faeces. Following overnight incubation at 4°C, solid material was disrupted by vortexing then centrifuged (25,000g, 6 min). The supernatant was stored at -20°C in pre-blocked eppendorfs.

35 To collect saliva samples anaesthetized mice were injected with 200µl of 20µg/ml carbachol in PBS to induce salivation.

Bronchoalveolar fluid was collected from killed mice. The throat region was exposed and muscle tissue surrounding the trachea removed. A small hole was made in the trachea and a lavage tip attached to a 1ml Tuberculin syringe containing 0.4ml of wash solution (1% v/v foetal calf serum in PBS) was inserted. After dispensing wash solution into the lungs, a 10 second rib-cage massage was performed prior to retraction of the syringe plunger and the extraction of lung fluid. Two more washes were performed using 0.3ml of wash solution.

10 *Detection of MV-specific antibodies*

Enzygnost measles-coated plates (Dade-Behring, Germany), containing simian kidney cells infected with MV, were used for detection of anti-MV antibody in mouse samples. MV-specific antibodies were detected with peroxidase-conjugated goat anti-mouse IgG followed by tetramethyl-bromide (TMB) substrate.

IgG-typing was performed using alkaline phosphatase (AP) -conjugated anti-mouse IgG1 or AP-conjugated anti-mouse IgG2a and *p*-Nitrophenyl phosphate (pNPP) substrate.

20 Mouse serum, salivary, BAL and faecal samples were assayed for the presence of IgA using AP-conjugated goat anti-mouse IgA with pNPP substrate.

Plaque reduction neutralization assay

25 The plaque reduction neutralization (PRN) titre is the reciprocal of the serum dilution capable of preventing 50% plaque formation by wild-type MV. The Edmonston strain of MV was used for this assay.

Four-fold dilutions of heat inactivated sera were prepared in supplemented RPMI (1/4 to 1/4096) and added to an equal volume of MV (200pfu/100µl). This serum/virus suspension was incubated at 37°C for 90 minutes before addition to 24-well, flat-bottomed plates containing 80% confluent RMK cells. Following a 90 minute incubation at 37°C 1ml/well of supplemented RPMI medium was added and plates were incubated at 37°C in a humidified atmosphere of 5% CO₂ for 72 hours.

35 Growth medium was removed and cells were fixed and permeabilised with 1ml/well of 10% formaldehyde with 0.1% Triton-X 100 in PBS for 20 minutes at RT. Plates were blocked with goat serum and anti-MV IgG

positive human serum was added. Anti-MV human sera was detected with FITC-conjugated anti-human IgG and fluorescing cells were examined using a Leitz fluovert inverted fluorescent microscope. Each cluster of fluorescing, infected cells was counted as one pfu. The serum dilution capable of preventing 50% plaque formation was generated according to the Karber formula.

Results

Transgenic tobacco plants producing MV-H protein

A 1.8kb fragment encompassing the coding region of the MV hemagglutinin (H) gene (Edmonston strain) was cloned into a plant expression cassette (Figure 1). To compare the effect of intracellular targeting on antigen yield, two additional clones were constructed, with a C-terminal SEKDEL sequence, coding for retention in the ER (pBinH/KDEL; Munro and Pelham 1987), and an authentic N-terminal plant signal peptide (pBinSP/H/KDEL; Hammond-Kosack *et al.*, 1994).

A total of 90 primary transformant (T_0) lines were obtained which showed detectable levels of MV-H gene expression by northern blot analysis (data not shown). A comparison of the six highest expressing lines for each construct are shown in Figure 2A. Transgene expression was similar for all three constructs. The selected high expressors shown in Figure 2A were further analysed for level of recombinant MV-H protein by ELISA using a rabbit anti-measles polyclonal antibody (Figure 2B). Plants transformed with the pBinH construct produced small quantities of recombinant MV-H protein. However, addition of the C-terminal KDEL sequence resulted in much higher levels of MV-H protein accumulation in plants transformed with the pBinH/KDEL construct. Interestingly, addition of the Pr1a plant signal peptide appeared to inhibit MV-H protein production in pBinSP/H/KDEL lines relative to the H/KDEL transgenic lines. For tobacco lines containing constructs pBinH and pBinH/KDEL, there appeared to be a reasonable correlation between transgene expression level and MV-H protein production (compare Figures 2A & 2B).

Seed was collected from the pBinH/KDEL T_0 transgenic lines showing the highest levels of H production (12C, 8B & 39H), germinated on kanamycin and re-assayed for MV-H protein production. ELISA analysis using the rabbit anti-measles polyclonal antiserum showed that the

introduced MV-H transgene was stably inherited in the T₁ progeny (Figure 3). Recombinant MV-H protein could also be detected in leaf extracts of pBinH/KDEL T₁ progeny by human serum (Figure 3). This serum was obtained from a subject with a history of wild-type measles infection, who had tested positive for measles antibodies by ELISA. The human serum detected similar quantities of MV-H protein in T₁ plants as the rabbit anti-measles polyclonal antiserum (Figure 3), confirming that the plant-derived MV-H protein retained at least some of the antigenic regions present in the native MV-H protein.

Further evidence of the authentic antigenicity of the recombinant MV-H protein was its positive reaction with two out of three MV-H protein monoclonal antibodies as tested by indirect ELISA. MAb-366 detected MV-H protein in extracts of pBinH/KDEL 8B (T₁) line with absorbance readings ranging from 0.392 to 0.420, compared to 0.018 to 0.019 for extracts from pBin control transgenic. The response of MAb-CV4 provided absorbance values ranging from 0.063 to 0.065 for the pBinH/KDEL extracts, compared to -0.005 to -0.001 for control transgenic extracts.

Intraperitoneal vaccination with plant-derived MV-H protein induces MV neutralizing antibodies

To determine the immunogenicity of the plant-derived MV-H protein groups of BALB/c mice were inoculated intraperitoneally with AS-purified plant extract from MV-H or control transgenic plants. Mice were inoculated on day 0, 14 and 49 and serum was collected on day 28 and 84. Significantly more MV-specific IgG was detected in mice vaccinated with plant-derived MV-H than in mice inoculated with control plant extract ($P < 0.01$) (Figure 4A). The MV-specific IgG was able to neutralize wild-type MV *in vitro* (Figure 4B). These results demonstrate that plant-derived MV-H protein is immunogenic when administered intraperitoneally.

Oral vaccination with plant-derived MV-H protein induces neutralizing antibodies and sIgA

Mice gavaged with either AS-purified MV-H or pellet MV-H extract have developed neutralizing antibodies to wild-type MV, details of one of these experiments are given below.

Groups of three mice were given 1g, 2g or 4g of plant extract containing the mucosal adjuvant CT-CTB by gavage on days 0, 7, 14, 21 and 35. Sera were collected on days 0, 7, 14, 21, 28, 49 and 78 and faecal isolates obtained on days 0 and 28. MV-specific serum IgG was only detected in groups that received 2g or 4g of MV-H plant extract. The serum IgG responses persisted for at least 78 days in mice gavaged with 2g of extract, but for only 49 days in mice gavaged with 4g of extract, with maximum titres of 2187 and 9 respectively. The lower response to 4g may be due to the increased dose to tobacco toxins also received.

High neutralizing ability was observed in pooled sera collected from mice gavaged with 2g of MV-H plant extract (Figure 5A). It peaked at 78 days with a PRN titre of 600. Mice gavaged with 4g of MV-H plant extract had a maximum neutralization titre of 150 at day 49. No neutralizing ability was detected in mice gavaged with 2g of control plant extract.

MV-specific secretory IgA (sIgA) was detected in faecal samples from some mice gavaged with 2g of MV-H plant extract (Figure 5B). This is a particularly important result as mucosal immunity is the first line of defense against airborne pathogens such as measles.

Vaccination with MV-H DNA constructs induces MV-neutralizing antibodies

Groups of five mice were injected with 100µg of MV-H DNA, or ovalbumin DNA (control) on day 0. Sera was collected on days 0, 15, 43 and 140, and faecal samples were obtained on days 0, 7, 14 and 21. Ten days after vaccination an increase in MV-specific IgG was only observed in the experimental group that received MV-H DNA. High serum IgG levels were maintained from day 20 to day 43, with a maximum titre of 729. In contrast to mice immunized with control DNA, which produced no MV-specific immune response, serum IgG from mice primed with MV-H DNA was able to neutralize wild-type MV *in vitro* (Figure 6). A neutralization titre of 900 was recorded at day 140, suggesting that the immune response is persistent. High titres of MV-neutralizing antibodies have previously been raised using MV-H DNA vaccines in mice (Yang *et al.* 1997, Polack *et al.* 2000), however some studies suggest that maternal antibodies may interfere with vaccine efficiency (Schlereth *et al.* 2000).

The predominant isotype present in mice immunized with MV-H DNA was IgG1, indicating a T_H2-type response. While intramuscular DNA

vaccines are generally associated with T_H1 -type responses, T_H2 dominated responses have been reported to occur in response to intramuscular DNA vaccination with a secreted form of measles H protein and a secreted hemagglutinin-based influenza DNA vaccine (Cardoso *et al.* 1996, Deliyannis *et al.* 2000). It is possible that this switching of IgG isotypes is due to a difference in antigen presentation when the encoded antigen is released from, rather than retained within, transfected cells, although there are no conclusive data to account for these differences.

No MV-specific serum or secretory IgA was detected in any DNA immunized group.

Oral delivery of MV-H protein following MV-H DNA vaccine boosts serum IgG titres

Mice were primed with 50 μ g of MV-H or control DNA on day 0. On days 21, 28, 35 and 42, these mice were boosted with 2g of either control or H protein plant extract, administered with CT-CTB. Sera were collected on days 0, 21 (pre-boost), and 49 (post-boost), and faecal isolates were obtained weekly until day 49. Salivary and bronchoalveolar lavage (BAL) samples were collected on day 49. Five mice were used per treatment.

MV-specific serum IgG titres were determined for pre-boost and post-boost pooled sera (Figure 7). Mice primed with MV-H DNA, produced MV-specific IgG, but mice given control DNA did not. The titre of the MV-H DNA IgG response was increased three-fold following gavage with MV-H plant extract. MV-H DNA primed mice boosted with control plant extracts also had higher post-boost IgG titres. However the absence of MV-specific serum IgG in mice primed with control DNA and boosted with control plant extract indicates that this is due to a continuing response to the MV-H DNA vaccine and not to the control plant extract. Delivery of the MV-H DNA vaccine followed by an oral MV-H plant boost resulted in higher serum IgG titres than either DNA vaccination or oral plant vaccination alone (MV-H DNA-control plant and control DNA - MV-H plant respectively).

Oral delivery of MV-H protein following MV-H DNA vaccine boosts neutralization titres

Neutralization assays were performed on pooled sera collected prior to DNA vaccination (day 0), immediately before boosting with plant extracts

(day 21) and 1 week after the final plant feeding (day 49) for each of the four treatment groups.

The neutralization titres exhibited similar trends to the IgG titres (Figure 8). At day 21 (pre-boost) serum from MV-H DNA primed mice had an average neutralization titre of 1150 compared to a titre of 8 for mice primed with control DNA. Following gavage with MV-H plant extracts neutralization titres increased relative to titres for mice boosted with control plant extract (Figure 8). The neutralization titre for MV-H DNA primed mice boosted with control plant dropped from 1150 to 450, while mice boosted with MV-H plant extract exhibited an increase in neutralization titre from 1150 to 2550. This suggests that boosting with MV-H plant extract has enhanced both the magnitude and the persistence of the immune response.

As with serum IgG titres combining the MV-H DNA vaccine and MV-H plant extract resulted in a synergistic response producing neutralization titres in excess of those recorded for either DNA or plant extract alone (Figure 8).

The present invention demonstrates that MV-H protein can be expressed in transgenic material and that this recombinant protein is recognised by host antibodies produced in response to wild-type measles infection. Furthermore the present invention shows that mice immunized intraperitoneally, by gavage or by DNA-oral prime-boost all developed antibodies able to neutralize wild-type MV *in vitro* (Figures 4B, 5A, 8). Neutralization titres for serum IgG were greater following DNA-oral prime boost than when either DNA or plant extracts were used alone (Figure 8). Finally, oral immunization using plant-derived MV-H protein resulted in the production of measurable levels of MV-specific sIgA (Figure 5B).

The present study demonstrates that "DNA vaccination-oral prime-boost" vaccination strategy utilising transgenic organisms is a viable approach to new vaccines. The potential for inducing a mucosal immune response, and seroconversion in the presence of maternal antibodies are important advances of this vaccine strategy. Availability of the vaccine in an "edible" form as a constituent of a fruit or vegetable crop will also enhance vaccination coverage by providing an inexpensive and relatively heat-stable package for distribution. Such a vaccine will have the potential to enable rates of vaccination to reach the targets required for global eradication.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

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CLAIMS:

1. A method for inducing an immune response to an antigen in a subject, the method comprising administering to the subject DNA encoding the
5 antigen, and subsequently orally administering to the subject a composition comprising transgenic material, wherein the transgenic material comprises a DNA molecule encoding the antigen such that the antigen is expressed in the transgenic material.
- 10 2. A method as claimed in claim 1 in which the composition further comprises a mucosal adjuvant.
3. A method as claimed in claim 2 in which the mucosal adjuvant is cholera toxin β -subunits.
15
4. A method as claimed in any one of claims 1 to 3 in which the antigen is expressed in the transgenic material as a fusion protein.
5. A method as claimed in claim 4 in which the fusion protein comprises
20 the antigen C-terminally fused to the amino acid sequence SEKDEL.
6. A method as claimed in any one of claims 1 to 5 in which the transgenic material is a transgenic plant.
- 25 7. A method as claimed in claim 6 in which the transgenic plant is a fruit or vegetable.
8. A method as claimed in claim 6 in which the transgenic plant is selected from the group consisting of; tobacco, lettuce, rice and bananas.
30
9. A method as claimed in any one of claims 1 to 8 in which the antigen is selected from the group consisting of viral antigens, parasitic antigens and bacterial antigens.
- 35 10. A method as claimed in claim 9 in the which the antigen is from measles virus, the human immunodeficiency virus, or Plasmodium sp.

11. A method as claimed in claim 10 in which the antigen is selected from the group consisting of the measles virus H or F protein, or fragments thereof.
- 5 12. A method as claimed in claim 11 in which the antigen is the measles H protein.
13. A method as claimed in any one of claims 1 to 12 in which the DNA encoding the antigen is administered only once to the subject.
- 10 14. A method as claimed in any one of claims 1 to 12 in which the DNA encoding the antigen is administered to the subject on at least two occasions.
- 15 15. A method as claimed in any one of claims 1 to 14 in which the composition comprising transgenic material is orally administered only once to the subject.
- 16 16. A method as claimed in any one of claims 1 to 14 in which the composition comprising transgenic material is orally administered to the subject on at least two occasions.
- 20 17. A transgenic plant, the plant having been transformed with a DNA molecule, the DNA molecule comprising a sequence encoding a measles virus antigen such that the plant expresses the measles virus antigen.
- 25 18. A transgenic plant as claimed in claim 17 in the DNA molecule encodes a fusion protein.
- 30 19. A transgenic plant as claimed in claim 18 in which the fusion protein comprises the measles antigen C-terminally fused to the amino acid sequence SEKDEL.
20. A transgenic plant as claimed in any one of claims 17 to 19 in which the measles antigen is the measles H protein.

Figure 1

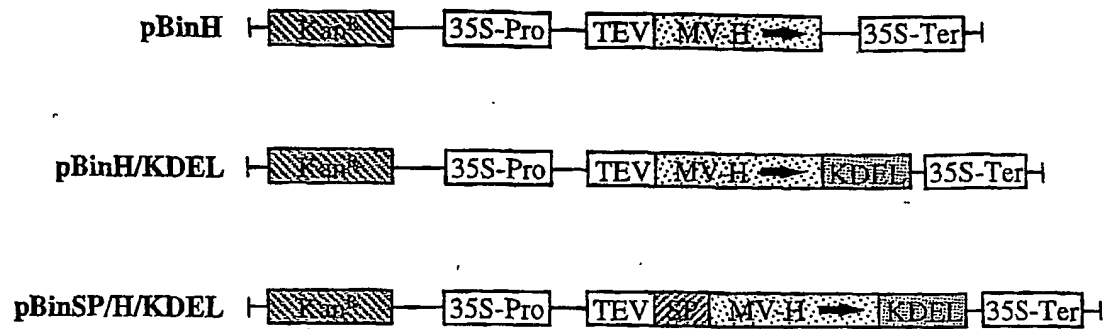


Figure 2

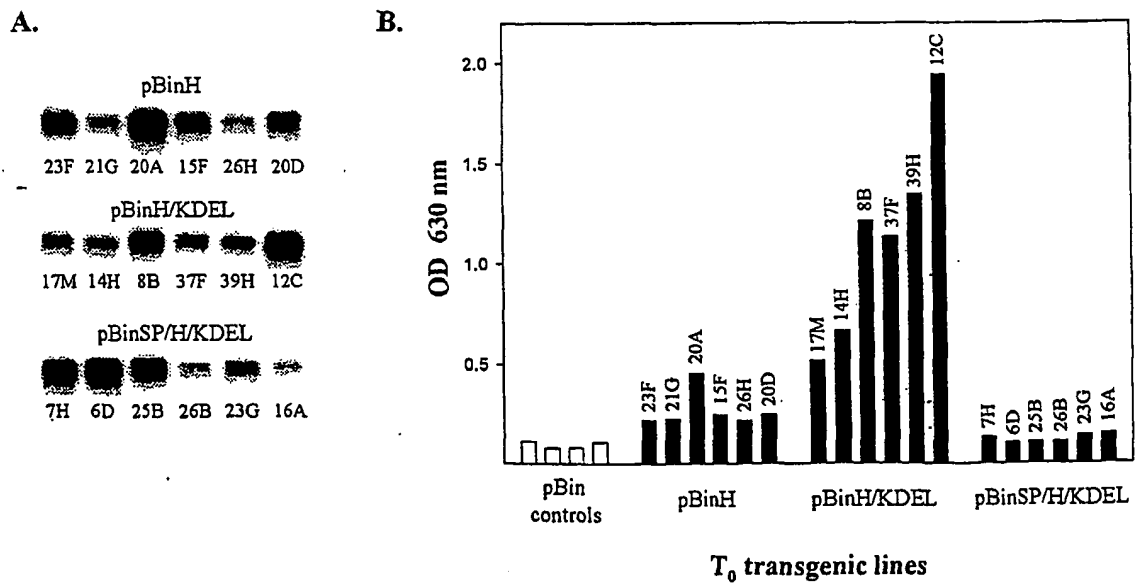


Figure 3

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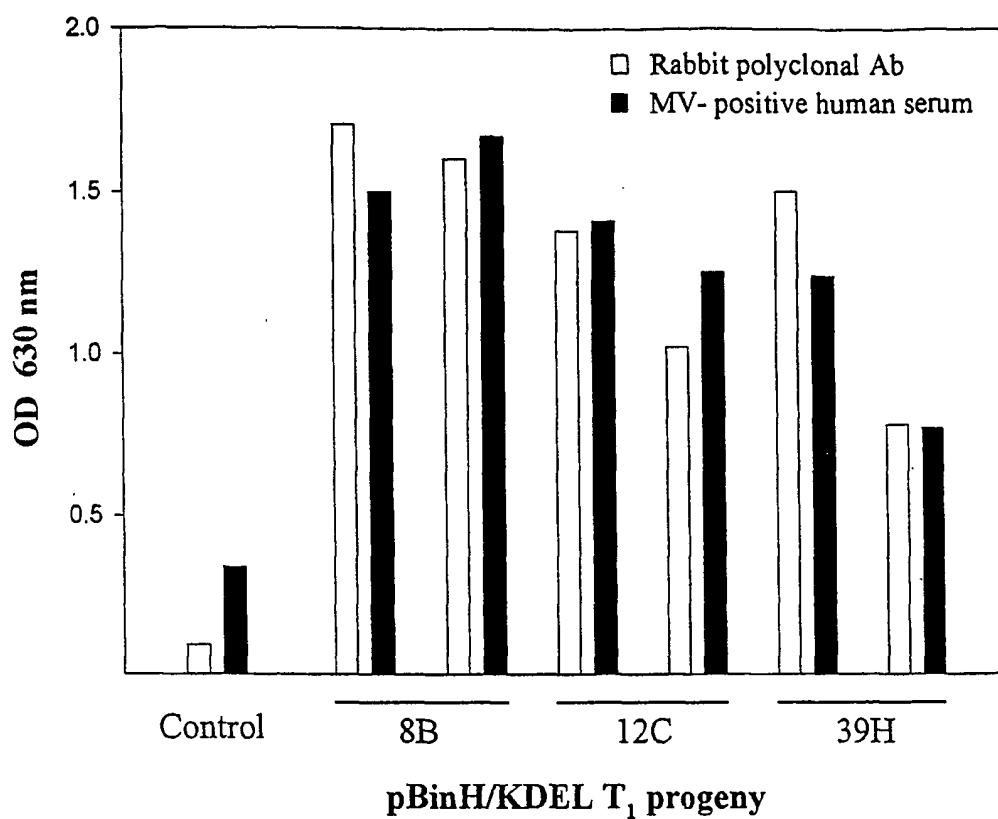
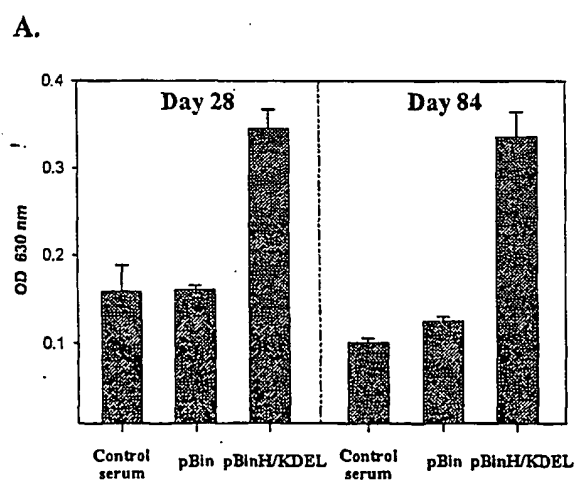


Figure 4



B.

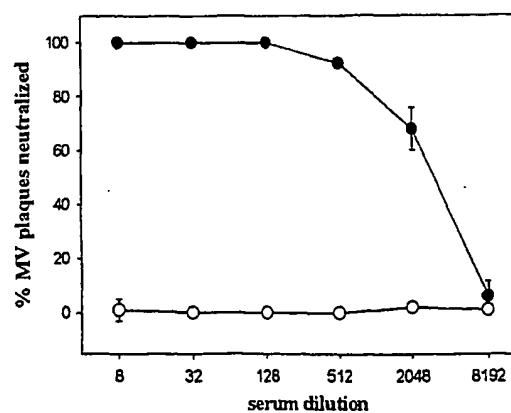


Figure 5

3/5

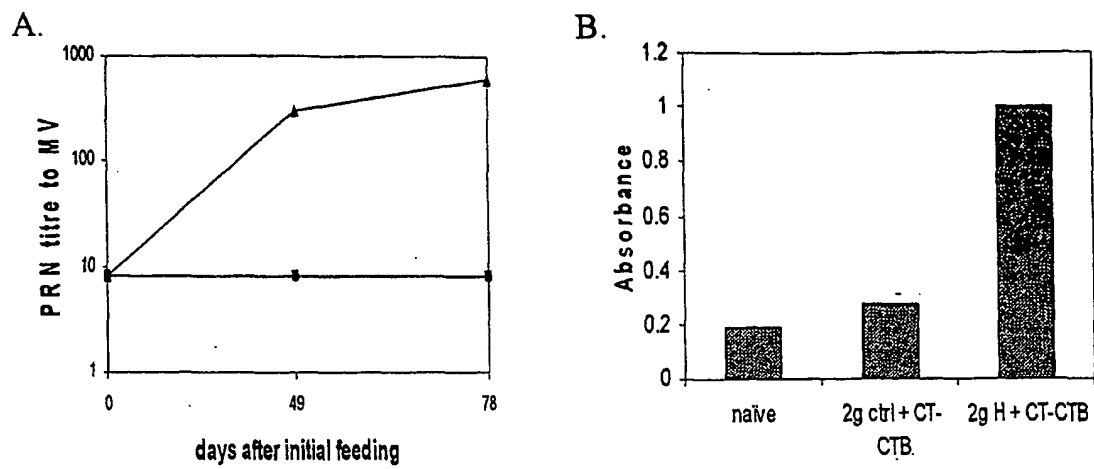


Figure 6

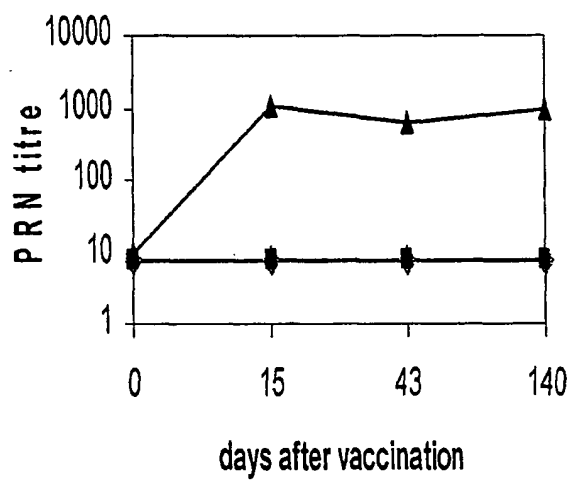
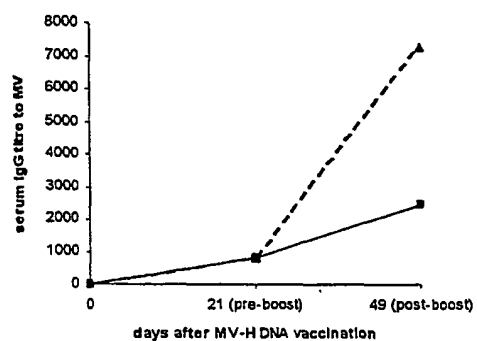
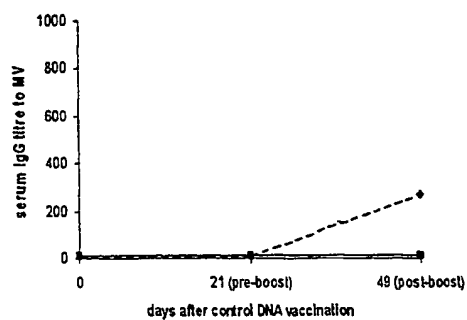


Figure 7

A



B

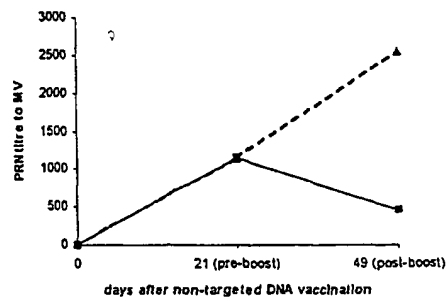


C

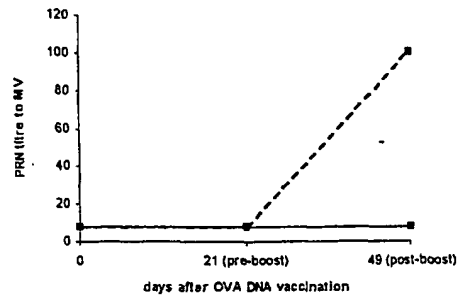
DNA vaccine	Plant feeding	Serum IgG titre		
		day 0	pre-boost (day 21)	post-boost (day 49)
MV-H	H	<3	810	7290
	Control			2430
control	H	<3	<3	270
	Control			<3

Figure 8

A.



B.



C.

DNA vaccine	Plant feeding	PRN titre		
		day 0	pre-boost	post-boost
MV-H	H	8	1150	2550
	Control			450
control	H	8	8	100
	Control			8

1/2

SEQUENCE LISTING

<110> Alfred Hospital
 Commonwealth Scientific and Industrial Research Organisation
 5 The University of Melbourne
 Australian National University

<120> Prime-boost vaccination strategy

10 <150> AU PQ 5208
 <151> 2000-01-21

<160> 7

15 <170> PatentIn Ver. 2.1

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 <211> 6
 20 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: ER retention signal
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 1 5

30

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35

<220>
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 - 40 tcgatctctg agaaagatga gctatgaggg 30

<210> 3
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 45 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: oligonucleotide

50

<400> 3
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55 <210> 4
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 <212> PRT

2/2

<213> Measles virus

<400> 4
Thr Asn Arg Arg
5 1

<210> 5
<211> 10
10 <212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: C-terminal end of measles H
15 protein fused with ER retention signal

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1 5 10
20

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<212> DNA
25 <213> Artificial Sequence

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<223> Description of Artificial Sequence: PCR primer

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<220>
40 <223> Description of Artificial Sequence: PCR primer

<400> 7
tatccatggg cccggcacgg caagagtggg atat 34
45

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU01/00059

A. CLASSIFICATION OF SUBJECT MATTERInt. Cl. ⁷: A61K 39/165; 35/78, A01H 5/00, A61K 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

FILE WPAT AND KEYWORDS BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

FILE MEDLINE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DERWENT WPAT and MEDLINE KEYWORDS: measles, measles (H) protein, antigen, transgenic() plant, immune, vaccine and DNA or gene

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Mahon B P et al. "Approaches to New Vaccines." Clinical Reviews in Biotechnology, Vol. 18(4), (1998), Pg 257-282. See whole document	1-20
X Y	Cardoso A I et al. "Immunization with Plasmid DNA Encoding for the Measles Virus Hemagglutinin and Nucleoprotein leads to Humoral and Cell-Mediated Immunity." Virology, Vol. 225, (1996) Pg 293-299. See whole document	1-20

☒ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

16 March 2001

Date of mailing of the international search report

3 APR 2001

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU01/00059

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Wigdorovitz A et al. "Induction of a Protective Antibody Response to Foot and Mouth Disease Virus in Mice Following Oral or Parenteral Immunization with Alfalfa Transgenic Plants Expressing the Viral Structural Protein VP 1". Virology, Vol. 255, (1999), Pg 347-353. See whole document	1-20
Y	WO 99/18225 A (LOMA LINDA UNIVERSITY) 15 April 1999 See whole document	1-20

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU01/00059

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO	9918225	AU	10724/99
END OF ANNEX			

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



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8 November 2001 (08.11.2001)

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(10) International Publication Number
WO 01/82962 A2

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- (21) International Application Number: **PCT/CA01/00577**
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- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (30) Priority Data:
60/200,011 **27 April 2000 (27.04.2000)** **US**
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- (74) Agent: **STEWART, Michael, L.**; Sim & McBurney, 6th Floor, 330 University Avenue, Toronto, Ontario M5G 1R7 (CA).
- (81) Designated States (national): **AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.**
- (84) Designated States (regional): **ARIPO** patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), **Eurasian** patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), **European** patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), **OAPI** patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— without international search report and to be republished upon receipt of that report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 01/82962 A2

(54) Title: **IMMUNIZING AGAINST HIV INFECTION**

(57) Abstract: A virus neutralizing level of antibodies to a primary HIV isolate is generated in a host by a prime-boost administration of antigens. The primary antigen is a DNA molecule encoding an envelop glycoprotein of a primary isolate of HIV-1 while the boosting antigen is either a non-infectious, non-replicating HIV-like particle having the envelope glycoprotein of a primary isolate of HIV-1 or an attenuated viral vector expressing an envelope glycoprotein of a primary isolate of HIV-1.

TITLE OF INVENTIONIMMUNIZING AGAINST HIV INFECTIONFIELD OF THE INVENTION

[0001] The present invention relates to the field of immunology and, in particular, to methods and compositions for immunizing a host against infection with HIV.

BACKGROUND OF THE INVENTION

[0002] Human immunodeficiency virus is a human retrovirus and is the etiological agent of acquired immunodeficiency syndrome (AIDS). It is estimated that more than 33 million people have been infected with HIV world-wide as of December 1999 (Ref 1- various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosure of these references are hereby incorporated by reference into the present disclosure).

[0003] As the HIV epidemic continues to spread world wide, the need for an effective vaccine remains urgent. Efforts to develop such a vaccine have been hampered by several factors three of which are: (a) the extraordinary ability of the virus to mutate; (b) inability of most known specificities of anti-HIV antibodies to neutralise HIV primary isolates consistently; and (c) lack of understanding of the correlates of protective immunity to HIV infection. Over the last 10 years, several candidate HIV vaccines have been tested in primates for their immunoprotective abilities (Ref. 2). These studies suggest that both neutralising antibodies and cell-mediated immunity play a role in conferring sterilizing immunity and preventing progression towards disease (Ref 3, 4). While the correlates for immune protection against HIV-1 infection are currently unknown, an effective HIV vaccine should elicit both strong neutralising antibody and cytotoxic T lymphocyte (CTL) responses.

[0004] Envelope subunit vaccines have been shown to induce high titred humoral responses, but were inefficient in eliciting CTL responses (Ref 5). Live recombinant pox vectors have been shown to elicit very potent CTL responses, however these vectors were ineffective for generating a significant antibody

response (Ref 6). In attempts to combine the two immunization types, several clinical trials involved a prime-boost strategy, consisting of initial viral vector immunization followed by boosts with recombinant HIV-1 envelope subunits (Ref 7, 8), have led to limited success with respect to CTL responses. Other vaccine approaches have used non-infectious, non-replicating, immunogenic virus-like particles (VLP) for immunising against HIV infection (Ref 9, 10). This type of immunogen has lead to the generation of neutralizing antibodies to a laboratory HIV-1 strain (Ref 10).

[0005] A prime-boost approach has been investigated using non-infectious VLPs to enhance HIV-specific CTL responses in mice primed with recombinant canarypox vector vCP205 encoding HIV-1gp 120 (MN strain) (Ref 11). This study showed that VLPs could boost the CTL response to the canarypox vector.

[0006] Recently, a study showing the induction of neutralizing antibodies to a HIV-1 primary isolate in chimpanzees has been reported (Ref 12). In this study, recombinant adenovirus expressing gp160 was used as the priming agent and recombinant gp120 protein was used to boost the monkeys.

[0007] There is still a need for vaccines and immunization regimes to induce both a strong CTL response as well as neutralizing antibodies to HIV primary isolates.

SUMMARY OF THE INVENTION

[0008] In accordance with one aspect of the present invention, there is provided a method for generating, in a host, particularly a human host, a virus neutralizing level of antibodies to a primary HIV isolate, comprising at least one administration of a priming antigen to the host, wherein the priming antigen comprises a DNA molecule encoding an envelope glycoprotein of a primary isolate of HIV, resting the host for at least one specific resting period to provide for clonal expansion of an HIV antigen specific population of precursor B-cells therein to provide a primed host, and at least one administration of a boosting antigen to the primed host to provide said neutralizing levels of antibodies, wherein the boosting antigen is selected from the group consisting of a non-infectious, non-replicating, immunogenic HIV-like particle having at least part of the envelope glycoprotein of a primary isolate of HIV and an attenuated viral

vector expressing at least part of an envelope glycoprotein of a primary isolate of HIV.

[0009] The primary HIV isolate may be an HIV-1 isolate including from the clade B HIV-1 clinical isolate HIV-1_{Bx08}, although any other primary HIV-1 isolate may be employed in the immunization procedures of the invention.

[0010] The DNA molecule encoding the envelope glycoprotein of a primary isolate of HIV may be contained in a plasmid vector under the control of a heterologous promoter, preferably a cytomegalovirus promoter, for expression of the envelope glycoprotein in the host, which may be a human host.

[0011] The vector utilized for DNA molecule immunization is novel and constitutes a further aspect of the present invention. Preferably, the vector has the identifying characteristics of pCMV3Bx08 shown in Figure 2, such identifying characteristics being the nucleic acid segments and restriction sites identified in Figure 2.

[0012] A priming administration of antigen may be effected in a single or in multiple administrations of the priming antigen. In the latter case, the at least one specific resting period to permit clonal expression of HIV antigen-specific population precursor B-cells may be effected after each priming administration. The at least one specific resting period may be between about 2 and 12 about months.

[0013] In the embodiment where the boosting antigen is a non-infectious, non-replicating, immunogenic HIV-like particle, such particle may comprise an assembly of:

- (i) an *env* gene product,
- (ii) a *pol* gene product, and
- (iii) a *gag* gene product

with the particle being encoded by a modified HIV genome deficient in long terminal repeats (LTRs) and containing *gag*, *pol* and *env* in their natural genomic arrangement. Such particles and the manufacture thereof are described in US Patent No. 5,439,809, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference. Such particles can include mutations in *gag* and *pol* to further reduce potential infectivity, as more fully described in United

States Patent No. 6,080,408, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference (WO 96/06177). In a preferred embodiment, the *env* gene is that from primary isolate BX08. The *gag* gene and *pol* gene may be those from the same primary isolate or may be chosen from those of other HIV-1 isolates, which may be primary isolates.

[0014] The non-infectious, non-replicating, immunogenic HIV-like particle may be administered in conjunction with an adjuvant. Any suitable adjuvant may be used, such as QS21, DC-chol, RIBI or Alum.

[0015] Such non-infectious, non-replicating, immunogenic HIV particle may be formed by expression from a suitable vector in mammalian cells. In accordance with an additional aspect of this invention, there is provided a vector comprising a modified HIV-genome deficient in long terminal repeats and a heterologous promoter operatively connected to said genome for expression of said genome in mammalian cells to produce the non-infectious, non-replicating and immunogenic particle, wherein at least the *env* gene of the modified HIV-genome is that from a primary isolate of HIV. The *gag* and *pol* genes of the modified HIV genome may be those from the same primary isolate or those from another isolate, which may be a primary isolate.

[0016] The vector preferably is a plasmid vector while the primary isolate preferably is BX08. The promoter may be the metallothionein promoter. The vector preferably has the identifying characteristics of plasmid p133B1 shown in Figure 3, such identifying characteristics being the nucleotide segments and restriction sites identified in Figure 3.

[0017] In the embodiment where the boosting antigen is an attenuated viral vector, the attenuated viral vector may be an attenuated avipox virus vector, particularly the attenuated canary poxvirus ALVAC. The attenuated viral vectors used herein form another aspect of the invention. The attenuated viral vector may contain a modified HIV genome deficient in long terminal repeats (LTRs), wherein at least the *env* gene is that from primary isolate BX08. The *gag* and *pol* genes of the modified genome may be those from the same primary isolate or may be chosen from other HIV isolate.

[0018] The attenuated canarypox virus-based vector ALVAC is a plaque-cloned derivative of the licensed canarypox vaccine, Kanapox, and is described in reference 19. The attenuated canary pox vector preferably has the identifying characteristics of vCP1579 shown in Figure 4, such identifying characteristics
5 being the nucleic acid segments and restriction sites identified in Figure 4.

[0019] The at least one administration of a boosting antigen may be effected in a single administration or at least two administration of the boosting antigen.

[0020] The invention further includes compositions comprising the
10 immunogens as provided herein and their use in the manufacture and formulation of immunogenic compositions including vaccines.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] The present invention will be further understood from the following description with reference to the drawings, in which:

15 Figure 1 shows the details of the elements of plasmid pCMVgDtat vprBx08.

Figure 2 shows the details of the elements of plasmid pCMV3Bx08.

Figure 3 shows the details of the elements of plasmid p133B1.

20 Figure 4 shows the details of the insertions into ALVAC (2) to provide vector vCP1579.

Figures 5A and 5B contain a representation in time-line form of the immunization regime used wherein the study groups are described in Table 1. The numbers below the lines refer to weeks.

25 Figure 6 shows the immunoreactivity to HIV-1 antigens of the serum diluted 1:100 from the macaques immunized with the various preparations as described in Table 1.

Figure 7 shows the immunoreactivity to HIV-1 antigens of the serum diluted 1:1000 from the macaques immunized with the various preparations
30 as described in Table 1.

Figure 8 shows the details of the elements of pMPC6H6K3E3.

Figure 9 shows the details of the elements of pMPC5H6PN.

Figure 10 shows the details of the elements of pHIV76.

Figure 11 shows the nucleotide sequence (SEQ ID NO: 1) for the H6/HIV Pol/Nef epitope cassette in the ALVAC C5 site of vCP1579.

Figure 12 contains the nucleotide sequence of C6 region (coding strand SEQ ID NO: 16, complementary strand SEQ ID NO: 17, K3L amino acid sequence SEQ ID NO: 18, E3L amino acid sequence SEQ ID NO: 19).

GENERAL DESCRIPTION OF INVENTION

[0022] As noted earlier, the present invention involves administration of HIV antigens to elicit virus-neutralizing levels of antibodies against a primary HIV isolate.

[0023] A DNA construct was prepared incorporating the *env* gene from the primary isolate Bx08 under the control of the cytomegalovirus promoter and the construct, pCMV3Bx08, is shown in Figure 2. The construct pCMV3Bx08 is derived from plasmid pCMVgDtat'vpr'Bx08 seen in Figure 1. The DNA construct pCMV3Bx08 was used in a priming immunization step to a host, macaque monkeys being the animal model chosen.

[0024] Following the priming immunization step, which may be effected in one or more administrations of the DNA construct, the host is allowed to rest to provide for clonal expression of an HIV antigen specific population of precursor B-cells therein to provide a primed host.

[0025] The boosting administration is effected either with a non-infectious, non-replicating, immunogenic HIV-like particle (VLP) or an attenuated viral vector.

[0026] For this purpose, a VLP expression plasmid was constructed containing a modified HIV genome lacking long terminal repeats in which the *env* gene is derived from primary isolate BX08, wherein the modified HIV genome is under the control of a metallothionein promoter. The construct, p133B1, shown in Figure 3, was used to effect expression in mammalian cells of the non-infectious, non-replicating, immunogenic HIV-like particules, in which the *env* gene product is that from the primary isolate BX08.

[0027] In the case of the attenuated virus vector, a recombinant attenuated canarypox virus vector was constructed to contain the *env* gene from primary

isolate BX08. The viral vector vCP1579 (Figure 4) was prepared by a variety of manipulations from plasmid pHIV76 (Figure 10), as shown described in detail below.

[0028] These products were utilized in a boosting administration to the primed macaques. The boosting administration may be effected in one or more immunizations. In a preferred aspect of the invention, the non-infectious, non-replicating immunogenic HIV-like particles may co-administered with the DNA construct in the priming administration and the DNA construct may be coadministered with the HIV-like particles in the boosting administration.

10 [0029] Immunizations were effected in accordance with the procedure of the invention and the results obtained were compared with those obtained using other protocols according to the protocols set forth in Table 1. The immunization regimes used are shown as time lines in Figures 5A and 5B.

[0030] The results obtained following the various protocols showed that, in particular, a primary DNA vaccination in combination with a boost from either the VLP or the attenuated canarypox virus enhanced the levels of neutralizing antibodies, as indicated by the reduction of detectable p24 levels in cells infected with primary HIV isolates.

Biological Deposits

20 [0031] Certain vectors that are described and referred to herein have been deposited with the American Type Culture Collection (ATCC) located at 10801 University Boulevard Manassas, Virginia 20110-2209, USA, pursuant the Budapest Treaty and prior to the filing of this application. Samples of the deposited vectors will become available to the public and all restrictions imposed or access to the deposits will be received upon grant of a patent based on this United States patent application or the United States patent application in which they are described. In addition, the deposit will be replaced if viable samples cannot be dispensed by the Depository. The invention described and claimed herein is not limited in scope by the biological materials deposited, since the deposited embodiment is intended only as an illustration of the invention. Any

30 equivalent of similar vectors that contain nucleic acids which encode equivalent or

similar antigens as described in this application are within the scope of the invention.

Deposit Summary

	<u>Plasmid</u>	<u>ATCC</u>	<u>Deposit Date</u>
5	pMT-HIV	40912	October 12, 1990
	pCMVgDtat ⁻ vpr ⁻	209446	November 11, 1997

EXAMPLES

[0032] The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

15 [0033] Example 1

[0034] This Example describes the construction of plasmid pCMV3BX08.

[0035] The plasmid, pCMV3BX08, contains sequence segments from various sources and the elements of construction are depicted in Figure 2.

[0036] The prokaryotic vector pBluescript SK (Stratagene) is the backbone of the plasmid pCMV3.BX08 and was modified by the replacement of the *Amp*^R with *Kan*^R gene and the deletion of the *fl* and the *LacZ* region. To achieve the desired modifications, the sequence between *AhdI* (nucleotide 2,041) and *SacI* (nucleotide 759) of pBluescript SK, which contains the *Amp*^R, *fl* origin and the *LacZ*, was deleted. A 1.2 kb *PstI* fragment from the plasmid pUC-4K (Pharmacia) containing the *Kan*^R gene, was blunt end ligated to the *AhdI* site of

25 pBluescript SK in a counter-clockwise orientation relative to its transcription. A 1.6 kb *SspI/PstI* DNA fragment containing the human cytomegalovirus immediate-early gene promoter, enhancer and intron A sequences (CMV) was ligated to the other end of the *Kan*^R gene so that the transcription from the CMV

30 promoter proceeds in the clockwise orientation. A synthetic oligonucleotide segment containing translation initiation sequence and sequences encoding the human tissue plasminogen activator signal peptide (TPA) was used to link the

CMV promotor and the sequences encoding the envelope gene of the primary isolate HIV-1_{BX08}.

[0037] The envelope gene from the HIV-1 primary isolate BX08 was isolated from the plasmid pCMVgDtat⁻vpr⁻Bx08 illustrated in Figure 1. The
5 plasmid pCMVgDtat⁻vpr⁻Bx08 was derived from the deposited plasmid pCMVgDtat⁻vpr⁻, the construction of which is described in copending United States Patent Application No. 08/991,773 filed December 16, 1997, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference, (WO 99/31250). The plasmid pCMVgDtat⁻vpr⁻Bx08 was derived by
10 substituting the BX08 envelope sequence from clade B HIV-1 clinical isolate HIV-1_{BX08} for the modified HIV genome sequence present in pCMVgDtat⁻vpr⁻. Plasmid pCMVgDtat⁻vpr⁻Bx08 was restricted with the restriction enzyme *Xho* I and made blunt ended with Klenow treatment. A *Not* I partial digestion was then performed and the resulting 6.3 kb fragment containing the *env* gene was isolated.
15 Plasmid pCMV3 (Invitrogen) was restricted with *Bam* HI and made blunt ended with Klenow treatment. The plasmid pCMV3 was then restricted with *Not* I and the resulting 4.4 kb fragment was isolated. The 6.3 and 4.4 kb fragments were ligated together to produce plasmid pCMV3BX08 (Figure 2).

[0038] The pCMV3BX08 construct was introduced into HB101 competent
20 cells according to manufacturer's recommendations (GibcoBRL). Correct molecular clones were identified by restriction and sequencing analysis and their expression of envelope glycoprotein was examined in transient transfections followed by Western blot analysis.

[0039] All DNAs used for immunizations were prepared using EndoFree
25 Plasmid Kit (Qiagen). For intramuscular immunizations either 3 mg or 600 µg of pCMVBX08, in 100 µl PBS was injected.

[0040] Proviral DNA for clade B HIV-1 clinical isolate HIV-1_{BX08} originated at Transgene (Strasbourg, France) and was isolated from genomic DNA of cells infected with the virus.

30 [0041] Example 2

[0042] This Example describes the construction of plasmid p133B1.

[0043] A Bx08 plasmid expression vector (p133B1, Figure 3) used to transfect the mammalian cells was engineered in several stages using pUC18 as the initial host plasmid. First, an 8.3-kbp fragment of HIV-1_{LAI} provirus encoding the gag, pol and env proteins was isolated. This fragment lacked the transcription regulatory elements and long terminal repeat elements from each end of the proviral genome to ensure the virus-like particles would be replication-incompetent. This fragment was linked to an inducible human type IIA metallothionein (MTIIA) promoter (Ref 13) and also to a Simian Virus 40 polyadenylation (polyA) addition/transcription termination sequence from plasmid pSV2dhfr (Ref 14). The modified fragment was then inserted into the pUC18 host vector. The resulting deposit expression construct, named pMT-HIV, was used to transfect into African green monkey kidney (Vero) and COS monkey kidney cells. The procedure for obtaining pMT-HIV is further described in the aforementioned US Patent No. 5,439,809. Both transfected cell lines produced non-replicating virus-like particles when induced with metal ions (Ref 15).

[0044] Two further modifications were made to the proviral DNA in pMT-HIV to provide additional safety features to protect human cells against recombination events with reverse-transcribed DNA:

- 1) inactivation of the RNA packaging sequences; and
- 2) deletion of a large section of the *pol* gene encoding reverse transcriptase and integrase.

[0045] To delete the first RNA packaging signal, part of the DNA corresponding to the untranslated leader sequence of the mRNA was replaced with synthetic DNA lacking a 25-bp motif corresponding to nucleotides 753-777 (the *psi* sequence). To inactivate the second RNA packaging signal, two adenosine residues within a *gag* gene zinc finger sequence were changed to thymidine residues. Each of these residue changes had the effect of replacing cysteine residues in a Cys-His array with a serine in the gene product.

[0046] The *pol* gene deletion was effected by replacing a 1.9-kbp fragment with synthetic DNA containing stop codons in all three reading frames. This prevented read-through translation of the residual integrase coding sequence on the 3' side of the deletion. The 1.9-kbp deletion in *pol* also eliminated the

expression of reverse transcriptase and integrase enzymes. However, the deletion left intact the gene encoding the viral protease, which is both an immunogenic component of HIV-1 virus particles and allows the expression of particles with processed gag antigens closely resembling native virions (Ref 16). The protease
5 also contains epitopes that are conserved across HIV-1 clades. The modifications described with respect to *gag* and *pol* genes are more fully described in the aforementioned United States Patent No. 6,080,408 (WO 96/06177).

[0047] Finally, the HIV-1_{LAI} *env* gene within pMT-HIV was replaced with that of HIV-1_{Bx08}. To effect this replacement, a 2440-bp fragment containing the
10 *env* gene of Bx08 was amplified by polymerase chain reaction (PCR) from cells infected with this isolate. The PCR product was then used to replace the corresponding region present in pMT-HIV. However, the incoming fragment from HIV-1_{Bx08} was 125-bp shorter than the original HIV-1_{LAI} region owing to a deletion in the untranslated region between the *env* gene stop codon and the
15 termination/polyA addition sequence. The resulting construct replaced all but eleven amino acid residues of the LAI envelope proteins gp120 and gp41. Of these eleven, only the first three differ between the LAI and Bx08 isolates, and these are all charge-conservative changes meaning the final expression vector (p133B1) encoded a nearly authentic HIV-1_{Bx08} *env* protein.

20 [0048] Example 3

[0049] This Example describes the production of HIV-like particles.

[0050] African green monkey kidney (Vero) cells were recovered and cultivated in Dulbecco's modified Eagle medium (DMEM) containing 10% v/v fetal bovine serum (FBS), referred to below as Complete Medium. At passage
25 141, the cells were transfected with p133B1 using the calcium phosphate method when at approximately 30% confluence. The cells were shocked with glycerol 8 hours after transfection. For this step, six 10-cm dishes containing approximately 3.0×10^6 cells each in 10.0 mL of Complete Medium were prepared. Each dish received 25.0 µg of expression vector and 2.0 µg of plasmid pSV2neo (Ref 17).
30 The pSV2neo contains a selectable marker gene conferring resistance to the antibiotic geneticin (G418). Two days after transfection, the cells from each dish

were recovered by trypsinization and replated into twenty-five fresh dishes in Complete Medium supplemented with 0.5 mg/mL of G418.

[0051] In total, 394 colonies were isolated from the dishes using cloning cylinders. Each colony was recovered by trypsinization and divided into two
5 cluster dish wells, one of the wells per clone was induced after reaching 50% to 90% confluence. Prior to induction, the wells were treated by replacing all the medium with fresh Complete Medium containing 10.0 μ M 5-azacytidine. After incubating for between 18 hours and 22 hours, the medium was removed and replaced with fresh DMEM containing 0.2% v/v FBS, 2.0 μ M CdCl₂ and 200.0
10 μ M ZnCl₂. The wells were incubated for a further 20 hours to 24 hours at which time samples of the medium were removed and tested by p24 ELISA.

[0052] The twenty highest-producing clones, based on the p24 titre, were chosen and cells from the corresponding uninduced wells were sub-cultured into one T-25 and one T-150 flask per clone. Both flasks were grown to confluence.
15 The cells from the T-150 were recovered by trypsinization and cryopreserved at passage number 145. The cells from the T-25 were recovered by trypsinization every 3 days to 4 days and maintained up to passage 153. The cells were induced as above and samples retested by p24 ELISA at two different passages prior to passage 153.

20 [0053] The two highest p24 producers were chosen and were recovered by trypsinization every 3 days to 4 days up to passage 163. Samples from the clones were tested by p24 and gp120 ELISA from passage 158 and by p24 ELISA at passage 163, to assess clonal stability. The most suitable of these two cell lines, named 148 to 391, was chosen for further sub-cloning. The clone nomenclature
25 defines the experiment number for this procedure, which was 148, and the number of the clone, which was number 391 of the original 394 isolated.

[0054] The vero cells were grown for approximately 100 h to 103 h and the medium was then replaced with growth medium containing 5-azacytidine. The bottles were then incubated for a further 20 h to 22 h, at which time the
30 medium was replaced with serum-free medium containing CdCl₂ and ZnCl₂. The bottles were then incubated for 29 h to 31 h, at which time the medium was harvested, pooled and stored at 2°C to 8°C prior to purification.

[0055] The next day after harvesting, the solution was clarified, concentrated and diafiltered against phosphate buffer. The concentrate was passed through a ceramic hydroxyapatite (type I) column and the run-through was collected. The run-through from two successive sublots was pooled together and
5 pumped onto a sucrose density gradient in a continuous zonal ultracentrifuge rotor. Pseudovirion-containing fractions were collected and pooled. The pooled pseudovirion fractions were diafiltered against PBS containing 2.5% sucrose to reduce the sucrose content, concentrated and diafiltered again. The material was sterile filtered using a 0.2 μ m filter. At this stage the materials was designated as
10 a purified sub-lot and were stored at 2 to 8°C.

[0056] The adjuvants were prepared separately and filter sterilized before filling in single dose vials. QS21 was stored at -20°C.

[0057] Example 4

[0058] This Example describes the production of recombinant pox virus
15 vCP1579.

[0059] Recombinant pox virus vCP1579 (Figure 4) contains the HIV-1 gag and protease genes derived from the HIV-1 IIIB isolate, the gp120 envelope sequences derived from the HIV-1 Bx08 isolate, and sequences encoding a polypeptide encompassing the known human CTL epitopes from HIV-1 Nef and
20 Pol.

[0060] Recombinant vCP1579 (Figure 4) was generated by insertion of the vector modifying sequences from pMPC6H6K3E3 (Figure 8) encoding E3L and K3L into the C6 site of recombinant vCP1566 (Figure 4). Recombinant vCP1566 was generated by insertion of an expression cassette encoding a synthetic
25 polypeptide containing Pol CTL epitopes and Nef CTL epitopes (Figure 11) and plasmid pMPC5H6PN (Figure 9) into vCP1453 at the insertion site known as C5. Recombinant vCP1453 was generated by co-insertion of genes encoding HIV-1 env and gag/protease gene products, plasmid pHIV76 (Figure 10), into the ALVAC genome at the insertion site known as C3.

30 [0061] The construction of recombinant pox vectors containing the E3L and K3L genes has been described in United States patent 6,004,777 issued Dec 21, 1999 to Tartaglia et al. and the recombinant pox vectors describing the

insertion of HIV genes has been described in United States patent 5,766,598 issued June 16 1998 to Paoletti et al.

- [0062] The locus designated C3 was used for the insertion of the HIV-1 *env* and *gag* gene sequences into the ALVAC(2) vector, and the locus designated as C5 was the insertion site for the sequences encoding the HIV-1 Nef and Pol CTL epitopes. By virtue of the C3 and C5 loci existing within the extensive inverted terminal repetitions (ITRs) of the virus genome (approximately 41 kbp), insertion into these loci results in the occurrence of two copies of the inserted HIV-1 sequences.
- 10 [0063] Briefly, expression cassette pHIV76 (Figure 10) was engineered in the following manner. Plasmid p133B1 (Figure 3) containing the HIV-1Bx08 gp 160 gene was used as the starting plasmid. The 3'-end of the H6 promoter was cloned upstream of the gp160 gene and three poxvirus early transcription termination signal sequences (T₃NT) were modified. This was accomplished by
- 15 cloning a 2,600 bp *Bam*HI-digested PCR fragment, containing the 3'-end of the H6 promoter and the T₃NT-modified HIV-1 (BX08) gp160 gene, into the *Bam*HI site of pBS-SK. This PCR fragment was generated from four overlapping PCR fragments (a 570 bp fragment, a 140 bp fragment, a 500 bp fragment and a 1,450 bp fragment) and the oligonucleotides, RW835 (5'-ATCATCATCGGATCC
- 20 CGGGGTCGCGATATCCGTTAAGTTTGTATCGTAATGAAAGTGAAGGAC C-3' - SEQ ID NO: 2) and RW836 (5'-ATCATCATCGGATCCCGGGGTT ATAGCAAAGCCCTTTC-3' - SEQ ID NO: 3). The 570 bp PCR fragment, containing the 3'-end of the H6 promoter and the 5'-end of the gp160 gene, was generated from the plasmid, p133B1, with the oligonucleotides, RW835 (5'-ATC
- 25 ATCATCGGATCCCGGGGTCGCGATATCCGTTAAGTTTGTATCGTAATG AAAGTGAAGGAGACC-3') and RW868 (5'-ATCAAGACTATAGAAGA GTGCATATTCTCTTTCATC-3'). The 140 bp PCR fragment, containing an interior portion of the gp160 gene, was generated from plasmid p133-B1 with the oligonucleotides, RW864 (5'-GCACTCTTCTATAGTCTTGATATAGTAC-3' -
- 30 SEQ ID NO: 4) and RW865 (5'-AGCCGGGGCGCAGAAATGTATG GGAATTGGCAC-3' - SEQ ID NO: 5). The 500 bp PCR fragment, containing an interior portion of the gp160 gene, was generated from 133-3 with the

oligonucleotides, RW866 (5'-ATACATTTCTGCGCCCCGGCTGGT
TTTGCGATTC-3' - SEQ ID NO: 6) and RW867 (5'-GAAGAATTC
CCCTCCACAATTAAAAC-3' - SEQ ID NO: 7). The 1,450 bp PCR fragment,
containing the 3'-end of the gp160 gene, was generated from p133-B1 with the
5 oligonucleotides, RW869 (5'-TGTGGAGGGGAATTCTTCTACTGTAATAC
AACACAAC-3' - SEQ ID NO: 8) and RW836 (5'-ATCATCATCGGAT
CCCGGGGTTATAGCAAAGCCCTTTC-3' - SEQ ID NO: 9). The 3'-end of the
570 bp PCR fragment overlaps the 5'-end of the 140 bp PCR fragment. The 3'-
end of the 140 bp PCR fragment overlaps the 5'-end of the 500 bp PCR fragment.
10 The 3'-end of the 500 bp PCR fragment overlaps the 5'-end of the 1450 bp PCR
fragment. The plasmid generated by this manipulation is called pRW997.

[0064] The sequence encoding gp41 was then replaced with the sequence
encoding the gp160 transmembrane (TM) region. This modification was
accomplished by cloning a 200 bp *MfeI-HindIII*-digested PCR fragment,
15 containing the 3'-end of the gp120 gene and the TM sequence, into the 4,400 bp
MfeI-HindIII fragment of pRW997. This PCR fragment was generated from two
overlapping PCR fragments (a 170 bp fragment and a 125 bp fragment) with the
oligonucleotides, HIVP97 (5'-TAGTGGGAAAGAGATCTTCAGACC-3' - SEQ
ID NO: 10) and HIVP101 (5'-TTTTAAGCTTTTATCCCTGCCTAACT
20 CTATTCAC TAT-3' - SEQ ID NO: 11). The 170 bp PCR fragment was
generated from pRW997 with the oligonucleotides, HIVP97 (5'-
TAGTGGGAAAGAGATCTTCAGACC-3' - SEQ ID NO: 12) and HIVP100 (5'-
CCTCCTACTATCATTATGAATATTCTTTTTTCTCTCTGCACCACTCT-3' -
SEQ ID NO: 13). The 125 bp PCR fragment was generated from pRW997 with
25 the oligonucleotides, HIVP99 (5'-AGAGTGGTGCAGAGAGAAAAA
AGAATATTCATAATGATAGTAGGAGGC-3' - SEQ ID NO: 14) and HIVP101
(5'-TTTTAAGCTTTTA TCCCTGCCTAACTCTATTCACTAT-3' - SEQ ID NO:
15). The plasmid generated by this manipulation is called pHIV71.

[0065] The H6-promoted gp120+TM gene was then cloned between C3
30 flanking arms, into a plasmid containing the I3L-promoted HIV1 gag/(pro) gene.
This modification was accomplished by cloning the 1,600 bp *NruI-XhoI* fragment
of pHIV71, containing the H6-promoted gp120+TM gene, into the 8,200 bp *NruI*-

*Xho*I fragment of pHIV63 . The plasmid generated by this manipulation is called pHIV76 (Figure 10). Plasmid pHIV76 was used in *in vivo* recombination experiments with ALVAC (CPpp) as rescue virus to yield vCP1453.

[0066] The sequence of the *nef/pol* regions is shown in Figure 12 and the
5 E3L and K3L sequences are shown in Figure 13. To generate ALVAC(2)120(BX08)GNP (vCP1579), expression cassettes consisting of the promoter/HIV-1 gene combinations were subcloned into an ALVAC donor plasmid, which were then used to insert the expression cassettes into defined sites in the ALVAC genome by *in vitro* recombination as previously described (Ref
10 20).

[0067] Example 5

[0068] This Example describes the results of immunization regimes.

[0069] Groups of four animals (macaques) each were randomly assigned to seven vaccine groups as illustrated in Table 1. In this Table, "BX08 DNA"
15 refers to pCMV3BX08, prepared as described in Example 1, "BX08 VLP" refers to the pseudovirions produced by expression vector p133B1 in Vero cells, as described in Example 3, and "ALVAC(2) BX08" refers vCP1579, prepared as described in Example 4. Reference (pre-bleed) sera were sampled at -6 and -2 weeks pre-vaccination. Primary immunizations with the various vaccines were
20 given on weeks 0 and 4 with boosts on weeks 24 and 44 (Figures 5A, 5B). The vaccines were immunized intramuscularly into one quadricep of each macaque monkey.

[0070] Sera were prepared from whole-blood using SST collection tubes and analyzed using commercially available HIV-1 western blots. Groups 1, 2 and
25 7 showed low levels of anti-Env antibodies after the first boost (Figures 6 and 7). Based on ELISA values, the anti-env antibody levels were below 1 µg/ml of specific IgG. High levels of anti-gag antibodies were detected in groups 1, 2, 3, 4, and 7 (Figures 6 and 7). No HIV-1 specific antibodies were detected in groups 5 and 6 (Figure 6).

30 [0071] The ability of the antibodies raised in the immunized monkeys to neutralize HIV-1BX08 virus in human PBMC was assayed based on the reduction of p24 levels.

[0072] The neutralization assay was performed essentially as described in reference 18. Briefly, serum dilutions were mixed with HIV-1 BX08 and the mixtures incubated for 1 hour, then added to susceptible human PBMC cells. Titres were recorded as the dilution of serum at which p24 was reduced by 80%.

5 Serum samples were assayed at 1:2, 1:8 and 1:32 dilution on the virus (1:6, 1:24 and 1:26 dilutions after the addition of cells). p24 levels were evaluated by p24-specific ELISA assay.

[0073] DNA vaccination on its own, group 5, and ALVAC on its own, group 6, had no monkeys showing reduction of p24 levels greater than 80%. The low DNA (600 ug) plus ALVAC, group 4, also showed no monkeys with greater than 80% reduction of p24 titres. VLP plus DNA, either high or low dose (group 1 and 2) showed enhanced reduction of p24 levels compared to VLPs alone, group 7. High dose DNA, group 3, in combination with ALVAC enhanced the ability to elicit p24 or virus neutralising antibodies over the low dose, group 4 or ALVAC alone, group 6. These results indicate that DNA vaccination in combination with VLPs or ALVAC enhanced the levels of virus neutralising antibodies as indicated by the reduction of p24 levels in the sera of the immunized monkeys.

[0074] The percentage reduction of p24 is calculated relative to the amount of p24 produced in the presence of the corresponding dilution of week 2 samples.

SUMMARY OF DISCLOSURE

[0075] In summary of this disclosure, the present invention provides novel immunization procedures and immunogenic compositions for generating virus neutralizing levels of antibodies to a primary HIV isolate and vectors utilized therein and for the generation of components for use therein. Modifications are possible within the scope of this invention.

Table 1 Study Design

Group number	Treatment – Week 0, 4	Treatment – Week 24,44
1	3 mg BX08 DNA 50 µg BX08 VLP 100 µg QS21	3 mg BX08 DNA 50 µg BX08 VLP 100 µg QS21
2	600 µg BX08 DNA 50 µg BX08 VLP 100 µg QS21	600 µg BX08 DNA 50 µg BX08 VLP 100 µg QS21
3	3 mg BX08 DNA	ALVAC(2) BX08 (1×10^8 pfu)
4	600 µg BX08 DNA	ALVAC(2) BX08 (1×10^8 pfu)
5	3 mg BX08 DNA	3 mg BX08 DNA
6	Control DNA	ALVAC(2) BX08 (1×10^8 pfu)
7	50 µg BX08 VLP 100 µg QS21	50 µg BX08 VLP 100 µg QS21

Table 2 Number of Monkeys showing > 80% reduction of p24 titre.

Group number	Week 26 Bleed	Week 44 Bleed
1	3/4	3/4
2	3/4	4/4
3	2/4	2/4
4	0/4	0/4
5	0/4	0/4
6	0/4	0/4
7	2/4	3/4

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CLAIMS

What we claim is:

1. A method for generating in a host a virus neutralizing level of antibodies to a primary HIV isolate, comprising:

at least one administration of a priming antigen to the host, wherein the priming antigen comprises a DNA molecule encoding an envelope glycoprotein of a primary isolate of HIV-1,

resting the host for at least one specific resting period to provide for clonal expansion of an HIV antigen specific population of precursor B-cells therein to provide a primed host, and

at least one administration of a boosting antigen to the primed host to provide said neutralizing levels of antibodies, wherein the boosting antigen is selected from the group consisting of a non-infectious, non-replicating, immunogenic HIV-like particle having at least the envelope glycoprotein of a primary isolate of HIV-1 and an attenuated viral vector expressing at least an envelope glycoprotein of a primary isolate of HIV-1.

2. The method of claim 1 wherein said primary isolate is Bx08.

3. The method of claim 2 wherein said DNA molecule is contained in a plasmid vector under the control of a heterologous promoter for expression of the envelope glycoprotein in the host.

4. The method of claim 3 wherein the promoter is the cytomegalovirus promoter.

5. The method of claim 4 wherein the vector has the identifying characteristics of pCMV3Bx08 shown in Figure 2.

6. The method of claim 1 wherein the at least one administration of a priming antigen is at least two administrations of the priming antigen.

7. The method of claim 6 wherein the at least one specific resting period is effected after each priming administration.

8. The method of claim 1 wherein the at least one specific resting period is between about 2 months to about 12 months.

9. The method of claim 1 wherein said non-infectious, non-replicating, immunogenic HIV-like particle comprises an assembly of:

(i) an *env* gene product,

- (ii) a *pol* gene product, and
- (iii) a *gag* gene product,

said particle being encoded by a modified HIV genome deficient in long terminal repeats (LTRs) and containing *gag*, *pol* and *env* in their natural genomic arrangement.

10. The method of claim 9 wherein the *env* gene is that from primary isolate BX08.

11. The method of claim 1 wherein said non-infectious, non-replicating, immunogenic HIV-like particle is administered in conjunction with an adjuvant.

12. The method of claim 11 wherein the adjuvant is QS21.

13. The method of claim 1 wherein said attenuated viral vector is an attenuated avipoxvirus

14. The method of claim 13 wherein the attenuated viral vector contains a modified HIV-genome deficient in long terminal repeats, wherein at least the *env* gene is that from primary isolate BX08.

15. The method of claim 14 wherein the attenuated avipoxvirus vector is the attenuated canary poxvirus ALVAC.

16. The method of claim 15 wherein the attenuated canary poxvirus vector has the identifying characteristics of vCP1579.

17. The method of claim 1 wherein the at least one administration of a boosting antigen is at least two administrations of a boosting antigen.

18. A vector, comprising a DNA sequence encoding an envelope glycoprotein of a primary isolate of HIV-1 under the control of a heterologous promoter for expression of the envelope glycoprotein in a host organism.

19. The vector of claim 18 wherein the vector is a plasmid vector.

20. The vector of claim 18 wherein said primary HIV-1 isolate is Bx08.

21. The vector of claim 20 wherein the promoter is the cytomegalovirus promoter.

22. The vector of claim 21 which has the identifying characteristics of pCMV3Bx08 shown in Figure 2.

23. The vector of claim 18 wherein the vector is an attenuated viral vector.

24. The vector of claim 23 wherein the attenuated viral vector is a attenuated avipoxvirus vector.
25. The vector of claim 24 wherein the attenuated avipoxvirus vector is the attenuated canary poxvirus vector ALVAC.
26. The vector of claim 25 wherein the attenuated viral vector has the identifying characteristics of vCP1579 shown in Figure 4.
27. A vector, comprising a modified HIV genome deficient in long terminal repeats and a heterologous promoter operatively connected to said genome for expression of said HIV genome in mammalian cells to produce non-infectious, non-replicating and immunogenic HIV-like particles, wherein at least the *env* gene is that from a primary isolate of HIV-1.
28. The vector of claim 27 wherein the vector is a plasmid vector.
29. The vector of claim 28 wherein the primary HIV-1 isolate is BX08.
30. The vector of claim 29 wherein the promoter is type IIA metallothionein promoter.
31. The vector of claim 30 which has the identifying characteristics of p133B1 shown in Figure 3.

Figure 1 Plasmid pCMV.Bx08.gp160

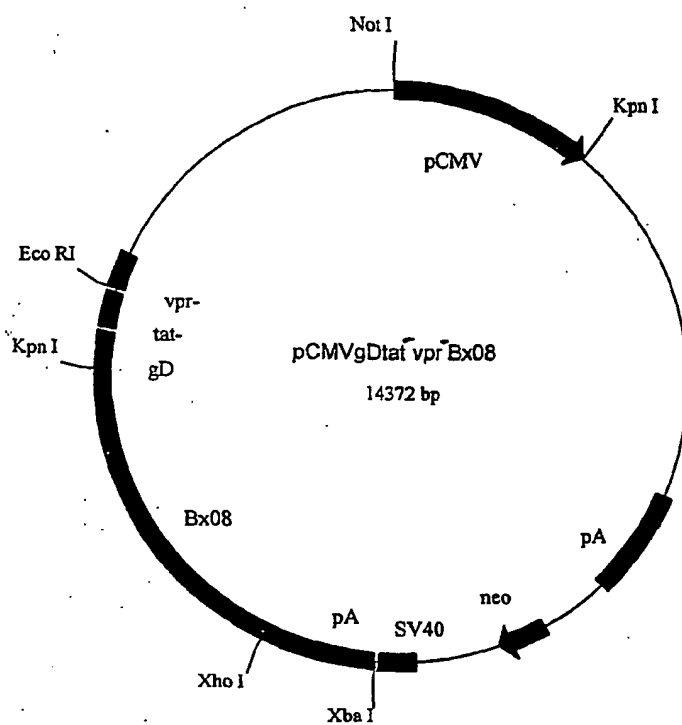


Figure 2 DNA immunization plasmid pCMV3Bx08.

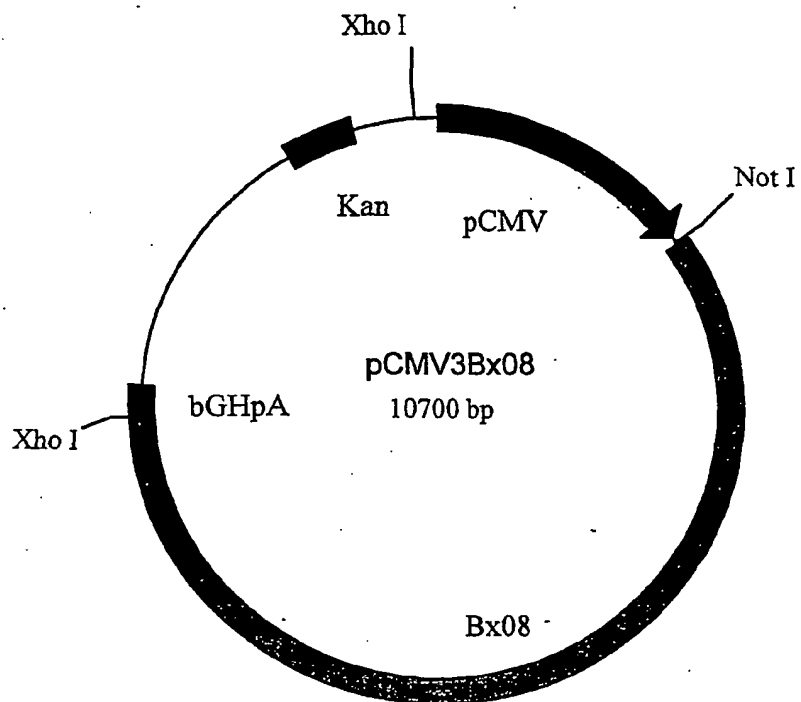


Figure 3. Pseudovirion Expression Plasmid p133B1 HIV-1 Bx08

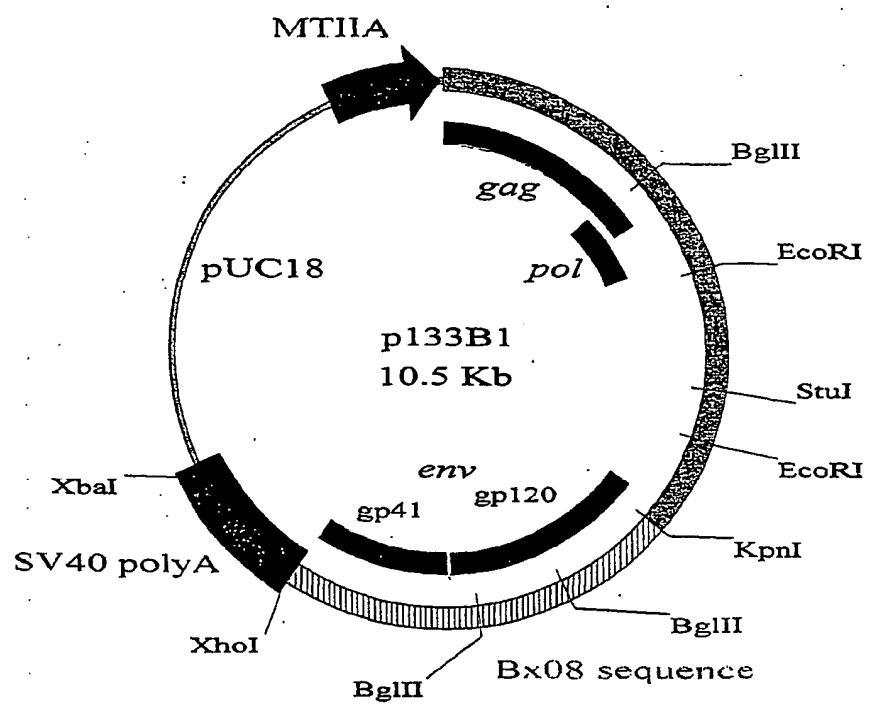


FIGURE 4
ALVAC(2)120(BX08)GNP
(vCP1579)
(ALVAC *Xho*I Restriction Map)

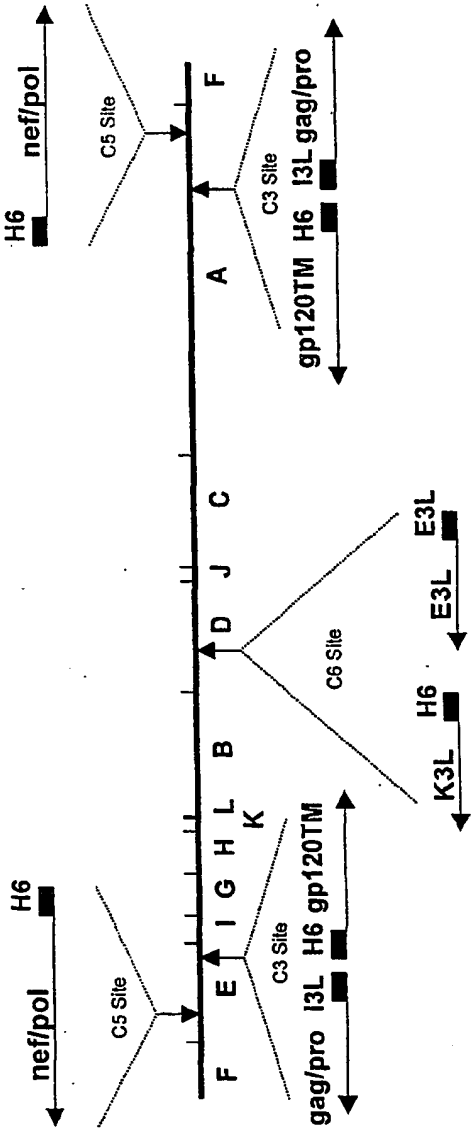


Figure 4: Time line of PM-C vaccine

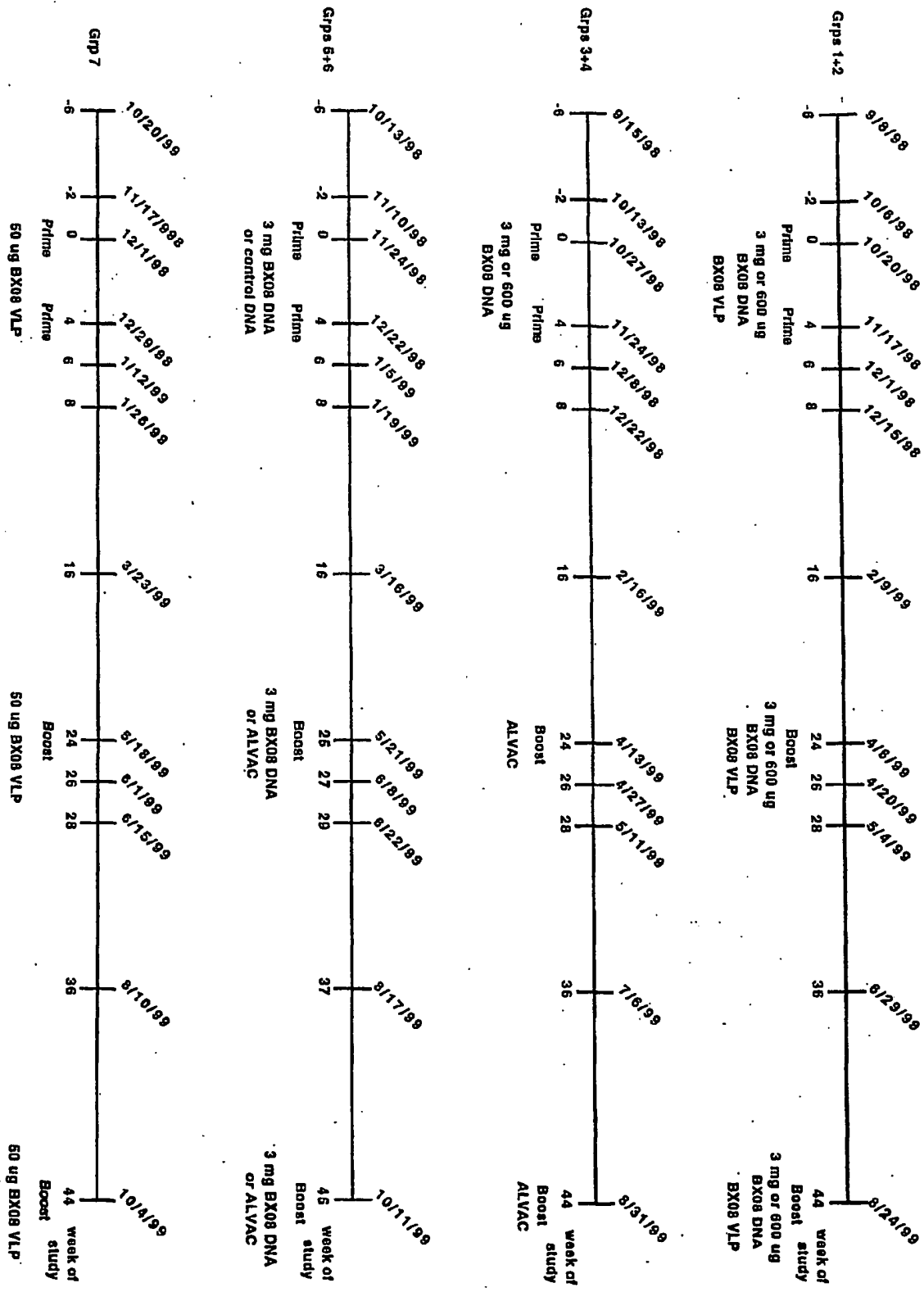
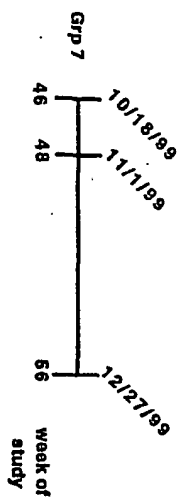
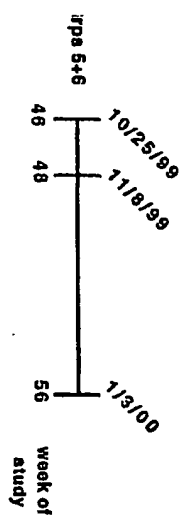
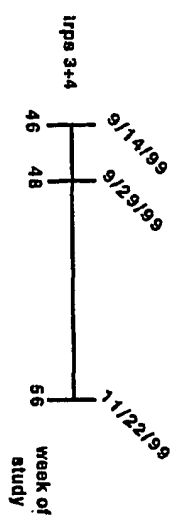
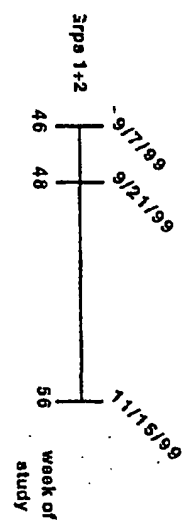


Figure 5 continued



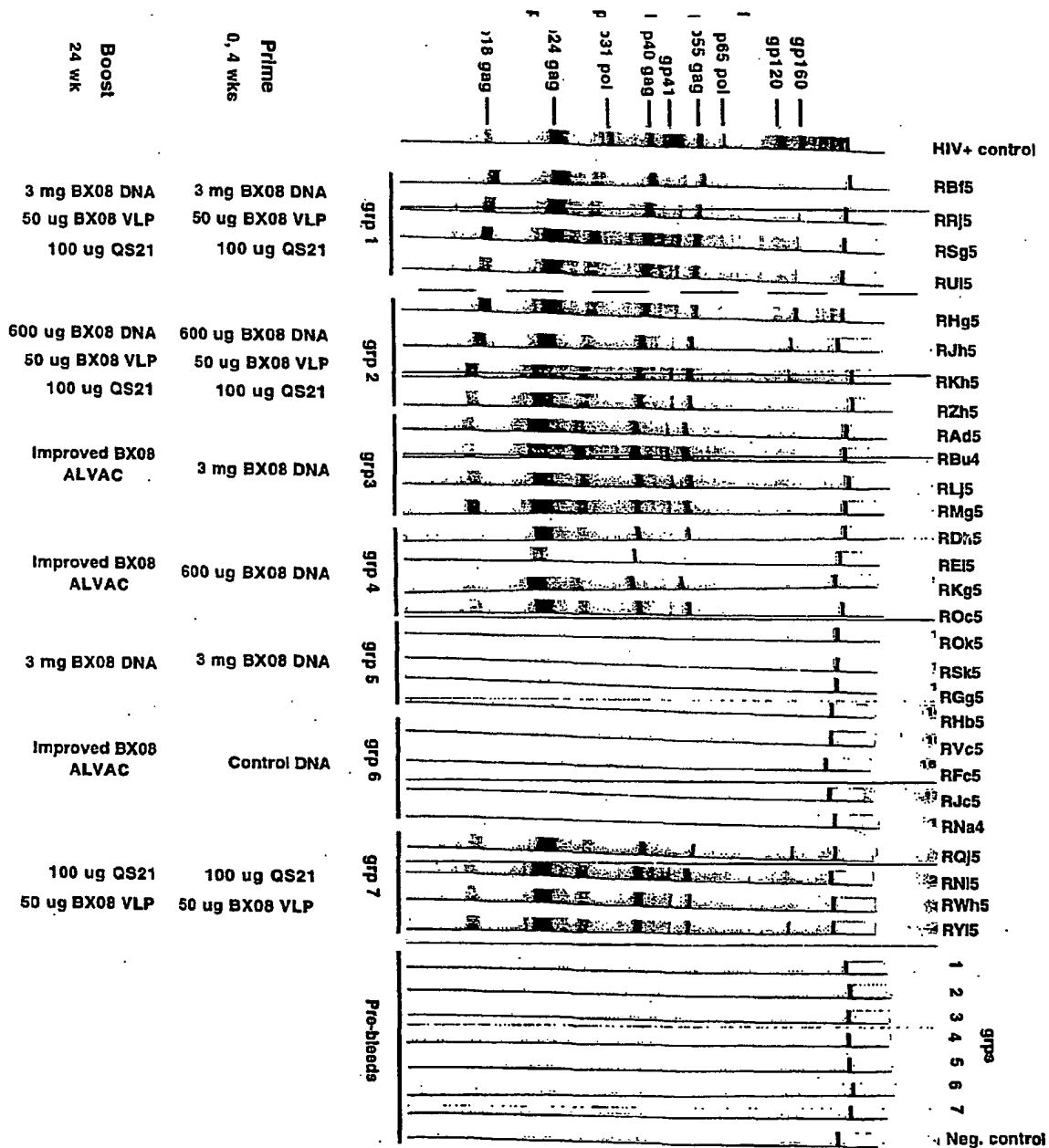
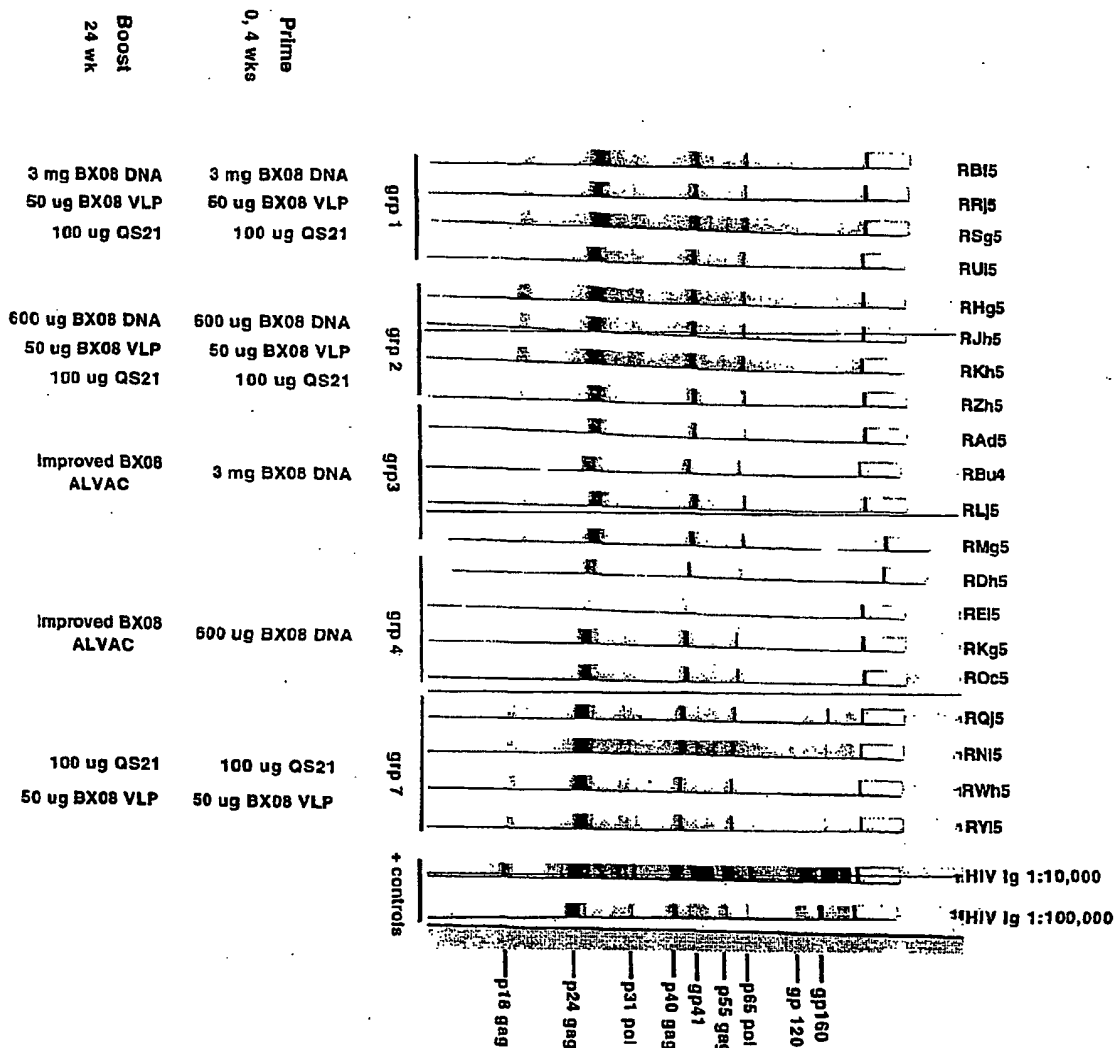
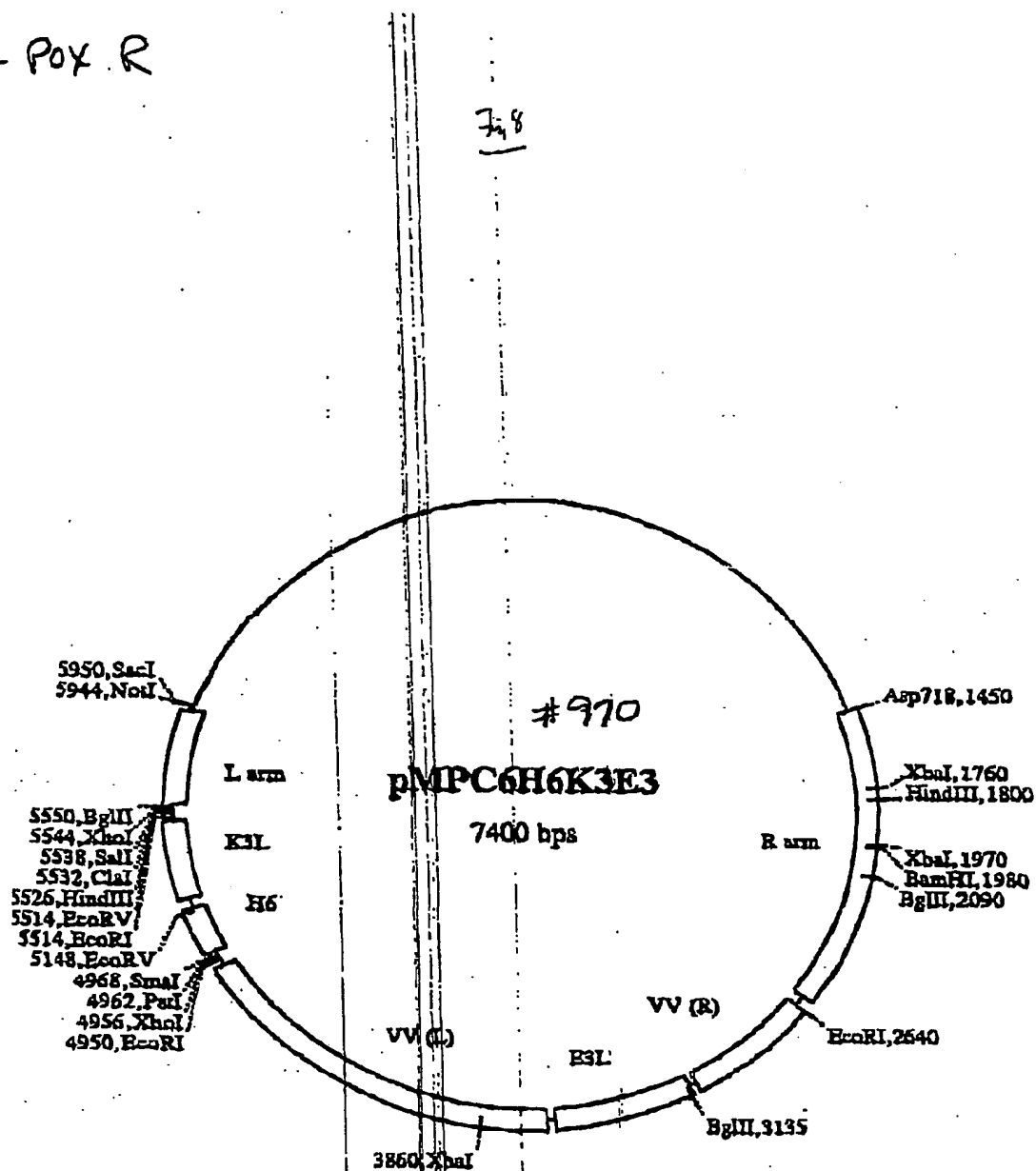


Figure 2. 26 wk macaque immunoreactivity to HIV antigens (1:100 diln)

647
Figure 3. 26 wk macaque serum immunoreactivity to HIV antigens (1:1000 diln)



2-14-Pox R



2-110-HIV

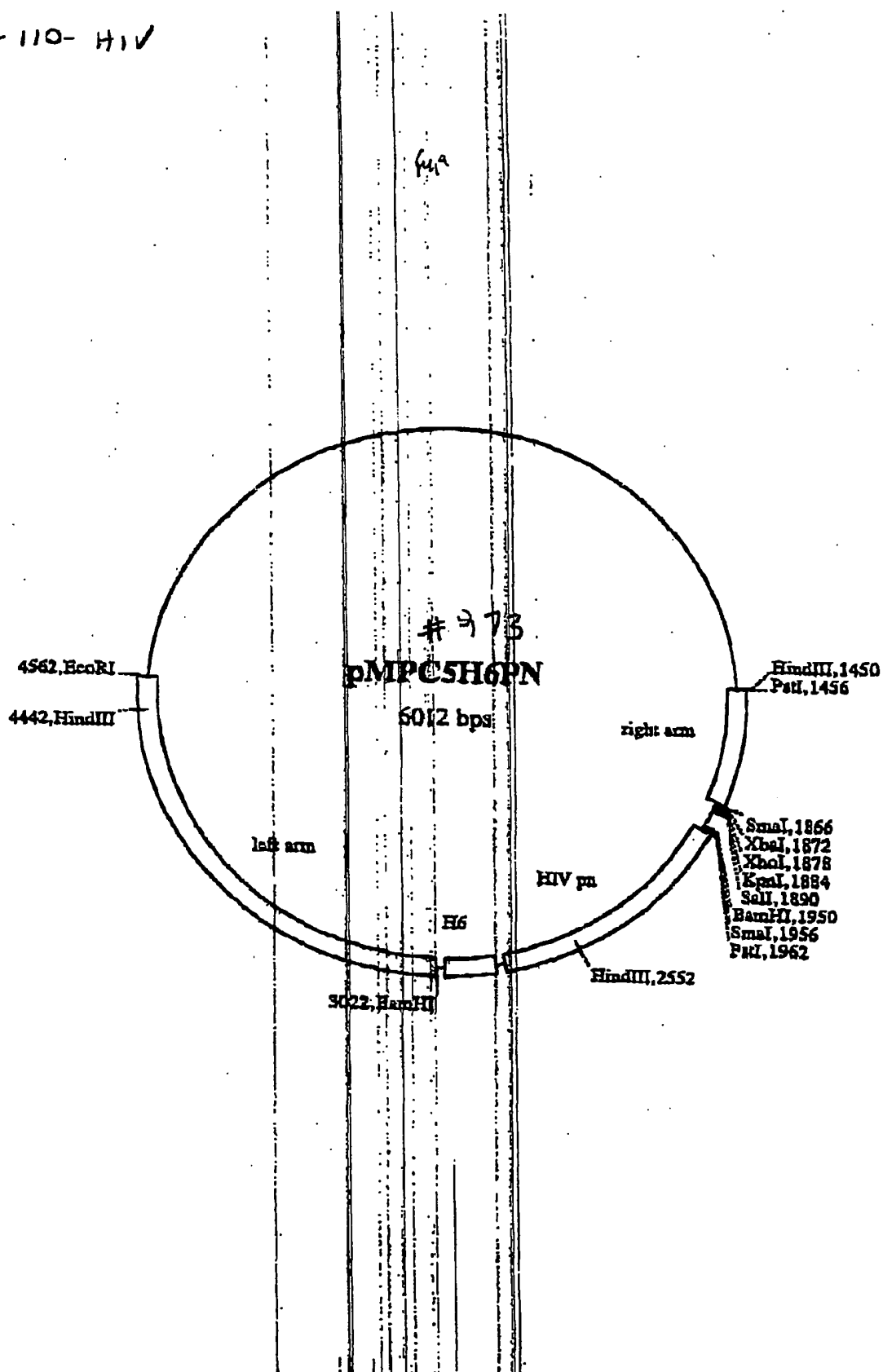


Figure 10 Plasmid pHIV76

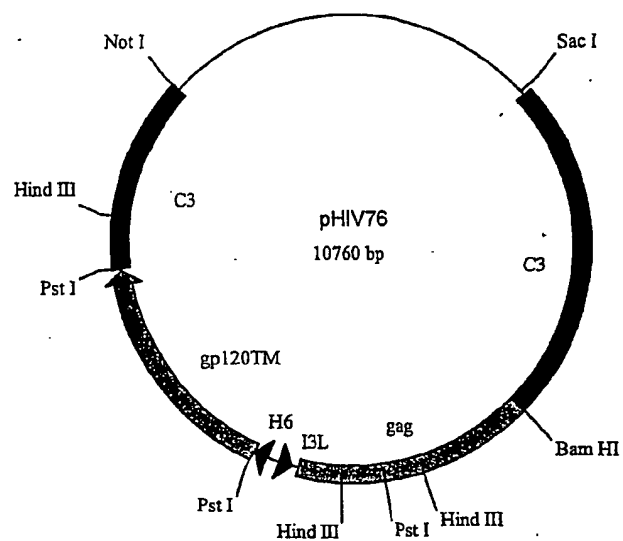


Figure 11

vCP1579: H6/HIV Pol/Nef epitope cassette in ALVAC C5 site

```
1 TTTTTTTCAT TATTTAGAAA TTATGCATTT TAGATCTTTA TAAGCGGCCG TGATTAACATA
61 GTCATAAAAA CCCGGGATCG ATTCTAGACT CGAGGGTACC GGATCTTAAT TAATTAGTCA
121 TCAGGCAGGG CGAGAACGAG ACTATCTGCT CGTTAATTAA TTAGGTGACG GGATCCCCCA
181 ACAAAAACATA ATCAGCTATC GGGGTTAATT AATTAGTTAT TAGACAAGGT GAAAACGAAA
241 CTATTTGTAG CTTAATTAAT TAGAGCTTCT TTATTCTATA CTTAAAAAGT GAAAATAAAT
301 ACAAGGTTTC TTGAGGGTTG TGTAAATTG AAAGCGAGAA ATAATCATAA ATTATTTTCAT
361 TATCGCGATA TCCGTTAAGT TTGTATCGTA ATGCCACTAA CAGAAGAAGC AGAGCTAGAA
421 CTGGCAGAAA ACAGAGAGAT TCTAAAAGAA CCAGTACATG GAGTGTATTA TGACCCATCA
481 AAAGACTTAA TAGCAGAAAT ACAGAAGCAG GGGCAAGGCC AATGGACATA TCAAATTTAT
541 CAAGAGCCAT TTA AAAATCT GAAAACAGGA ATGGAGTGGA GATTTGATTC TAGATTAGCA
601 TTTTCATCAG TAGCTAGAGA ATTACATCCT GAATATTTTA AAAATTGTAT GGCAATATTC
661 CAAAGTAGCA TGACAAAAAT CTTAGAGCCT TTTAGAAAAC AAAATCCAGA CATAGTTATC
721 TATCAATACA TGGATGATTT GTATGTAGGA TCTGACTTAG AAATAGGGCA GCATAGAACA
781 AAAATAGAGG AGCTGAGACA ACATCTGTTG AGGTGGGGAC TTACAACCAT GGTAGGTTTT
841 CCAGTAACAC CTCAGTACC TTTAAGACCA ATGACTTACA AAGCAGCTGT AGATCTTTCT
901 CACTTTTTTAA AAGAAAAAGG AGGTTTAGAA GGGCTAATTC ATTCTCAACG AAGACAAGAT
961 ATTCCTTGATT TGTGGATTTA TCATACACAA GGATATTTTC CTGATTGGCA GAATTACACA
1021 CCAGGACCAG GAGTCAGATA CCCATTAACC TTTGGTTGGT GCTACAAGCT AGTACCAATG
1081 ATTGAGACTG TACCAGTAAA ATTAAAGCCA GGAATGGATG GCCCAAAGT TAAACAATGG
1141 CCATTGACAG AAGAAAAAAT AAAAGCATTG GTAGAAATTT GTACAGAGAT GGAAAAGGAA
1201 GGGAAAATTT CAAAAATTGG GCCTTAATTT TTCTGCAGCC CGGGGGATCC TTTTATAGC
1261 TAATTAGTCA CGTACCTTTG AGAGTACCAC TTCAGCTACC TCTTTTGTGT CTCAGAGTAA
1321 CTTTCTTTAA TCAATTCCAA AACAG
```

Upstream (right) flanking sequence: 1-266

VV H6 promoter: 267-390

HIV pol/nef/pol/nef/pol cassette: 391-1227

Downstream (left) flanking sequence: 1227-1345

Figure 12

E3L and K3L genes in C6

```

      10      20      30      40      50      60      70      80      90     100     110
GAGCTGCGG CCCCTATCA AAGTCTTAA TGAGTTAGGT GTAGATAGTA TAGATATTAC TACAAAGGTA TTATATTTTC CTATCAATTC TAAAGTAGAT GATATTAATA
CTCGAGCGCC GCGGATAGT TTTCAGAAAT ACTCAATCCA CATCTATCAT ATCTATAATG ATGTTTCCAT AAGTATAAAG GATAGTTAAG ATTTTCATCTA CTATAATTAT

      120     130     140     150     160     170     180     190     200     210     220
ACTCAAAGAT GATGATAGTA GATAATAGAT ACGCTCATAT AATGACTGCA AATTGAGCG GTTCACATTT TAATCATCAC GCGTTCATAA GTTTCACCTG CATAGATCAA
TGAGTTTCTA CTACTATCAT CTATTATCTA TCGAGATATA TTAGTGACGT TTAACCTGCG CAAGTGTAAT ATTAGTAGTG CGCAAGTATT CAAAGTGAC GTATCTAGTT

      230     240     250     260     270     280     290     300     310     320     330
AATCTCACTA AAAAGATAGC CGATGTATTT GAGAGAGATT GGACATCTAA CTAGCGTAAA GAATTTACAG TTATAAATAA TACATAATGG ATTTTGTAT CATCAGTTAT
TTAGAGTAGT TTTTCTATCG GTACATATAA CTCTCTCTAA CCTGTAGATT GATGCGATTT CTTTAATGTC AATATTATTT ATGTATTACC TAAACAATAA GTAGTCAATA

      340     350     360     370     380     390     400     410     420     430     440
ATTTAACAATA AGTACATAAA AAAAGTATTAA ATAAAAATAC TTACTTACGA AAAATGACT AATTAGCTAT AAAAACCCAG ATCTCTCGAG GTCGAGCGTA TCGATAGCTT
TAAATGTATG TCAATGTATT TTTCATAATT TATTTTATG AATGAATGCT TTTTACTGTA TTAATGATA TTTTGGGTC TAGAGAGCTC CAGCTGCCAT AGCTATTGCA

      450     460     470     480     490     500     510     520     530
TGATATCGAA TTCATATAAA TT A TTG ATG TCT ACA CAT CCT TTT GTA ATT GAC ATC TAT ATA TCC TTT TGT ATA ATC AAC TCT ANT CAC TTT
ACTATAGCTT AAGTATTTTT AA T AAC TAC AGA TGT GTA GGA AAA CAT TAA CTG TAG ATA TAT AGG AAA ACA TAT TAG TTG AGA TTA GTG AAA
      <Q H R C M R K Y N V D I Y G K T Y D V R I V K
-----K3L-----

      540     550     560     570     580     590     600     610     620
AAC TTT TAC AGT TTT CCC TAC CAG TTT ATC CCT ATA TTC AAC ATA TCT ATC CAT ATG CAT CTT AAC ACT CTC TGC CAA GAT AGC TTC AGA
TTG AAA ATG TCA AAA GGG ATG GTC AAA TAG GGA TAT AAG TTG TAT AGA TAG GTA TAC GTA GAA TTG TGA GAG ACG GTT CTA TCG AAG TCT
<V K V T K G G V L K D R Y E V Y R D M H M K V S E A L I A E S
-----K3L-----

      630     640     650     660     670     680     690     700     710
GTG AGG ATA CTC AAA AAG ATA AAT GTA TAG AGC ATA ATC CTT CTC GTA TAC TCT GCC CTT TAT TAC ATC GCC CGC ATT GGG CAA CGA ATA
CAC TCC TAT CAG TTT TTC TAT TTA CAT ATC TCG TAT TAG GAA GAG CAT ATG AGA CGG GAA ATA ATG TAG CGG GCG TAA CCC GTT GCT TAT
<H P Y D F L Y I Y L A Y D K E Y V R G K I V D G A N P L S Y
-----K3L-----

      720     730     740     750     760     770     780     790     800     810
ACA AAA TGC AAG CAT ACG ATACAACTT AACGGATATC GCGATAATGA AATAATTAT GATTATTICT CGCTTTCAAT TTAAACAAC CCTCAAGAAC
TGT TTT ACG TTC GTA TGC TATGTTTGA TCGCTATAG CGCTATTACT TTATTAATAA CTATAAAGA GCGAAAGTTA AATTGTGTTG GGAGTCTGTG
      <C F A L M
-----K3L-----

      820     830     840     850     860     870     880     890     900     910     920
CTTTGTATTT ATTTTCACCT TTAAAGTATA GAATAAAGAA AGCTCTAATT AATTAAAGAA CAGATTGTTT CGTTTTCCTC TTGGCGTATC ACTAATTAT TAACCGGGC
GAACATATAA TAAAGTGAAG AAATTCATAT CTATTTCCTT TCGAGATTAA TTAATTACTT GTCTAACAAA GCAAAAGGGG AACCGCATAG TGATTAAATA ATTGGGCCCC

      930     940     950     960     970     980     990     1000    1010    1020    1030
TCGAGTCGCA GGAATTCAC TATATGCGCA TATTTCATT GTATACACAT AACCAATTACT AACGTAGAA GTATAGGAAG AGATGTAAGG GGAACAGGAT TTGTTGATTC
ACGTGAGCT CCTTAAGTTG ATATAGCTGT ATAAAGTAAA CATATGTGTA TTGTAATGA TTGCATCTTA CATATCCTTC TCTACATTGC CCTTGTCCCA AACCACTAAG

      1040    1050    1060    1070    1080    1090    1100    1110    1120    1130    1140
GCAACTATT CTAATACATA ATTCCTCTGT TAATACGCTT TGCAGTAAT CTATTATAGA TGCCAAAGATA TCTATATAAT TATTTTGTA GATGATGTTA ACTATGTGAT
CGTTTGATAA GATTATGAT TAAGAAGACA ATTATGCGAG ACGTGCATTA GATAATATCT ACGGTTCTAT AGATATATTA ATAAAAACAT CTACTACAAT TGATACACTA

      1150    1160    1170    1180    1190    1200    1210    1220    1230    1240    1250
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GATATATTCA TCACATATT AAGTACATATA AGCTATATATC AAGTTTGAGA CAGAAACACT ACAGATCAAA GCATTATAGA TATCGTAGGA GTTTTTATA TAAGCGTATA

      1260    1270    1280    1290    1300    1310    1320    1330    1340    1350    1360
ATTCCCAAGT CTTCACTCT ATCTCTTAAA AAATCTTCAA CGTATGGAAT ATAATATCT ATTTTACCTC TTCTGATATC ATTAATGATA TAGTTTTTGA CACTATCTTC
TAAGGGTTCA GAAGTCAAGA TAGAAGATTT TTAGAAGTT GCATACCTTA TATTATTAGA TAAATGGAG AAGACTATAG TAATTACTAT ATCAAAAACCT GTGATAGAAG

      1370    1380    1390    1400    1410    1420    1430    1440    1450    1460    1470
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ACAGTTAACT AAGAATAGT GATATAGATT CTTTGCCTAT CCGAGGGATC CTGCTTGATG ACGGTAATTA TAGAGATAAT ATCGAAGACC TGTATTAAAT AGATAATATG

      1480    1490    1500    1510    1520    1530    1540    1550    1560    1570    1580
CAGATTAAAT GGGACTATT CCGTATCTAT CTAACATAGT TTAAGMAAG TCAGAACTCA AGACCTGATG TTCAATATTT GTTTCATACA TGAATGATC TCTATTGATG
GTCTTAATTA CCTTGATAA GGCATAGATA GATTGTATCA AAATCTTTC AGTCTTAGAT TCTGGACTAC AAGTATATAA CCAAGTAGTT ACTTTACTAG AGATAACTAC
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TATCACTGAT AAGTAAGAG ACTTTTAACC ATTGAGTAAG ATATATAAGA AAGGAACAC TACTTCTAT CTTATATAG TATCTTAA TACGGTTGTT TGACAAGAGA

1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
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ATATCTAGCA TATAGTAGTA GACTTTATTA GTACATTCCG TATGTAAATT GTTAATCTCT GAACAGAGGA CAATAGTTAT ATGATAAGAA CACTATTAAA TACACACTCC

1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910
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GTTAAACAG GTGCAAGAA TTAACAAT ATCATCTATA GTTAGGTTA CTTCATGTC AAGAACCAG TTTGTCTATA TCAAAAGAC CTGTTTAA AGTTGTAT

1920 1930 1940 1950 1960 1970 1980 1990 2000 2010 2020
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AATATTCTT GAAACCATC TATTCACCT ACTTAGGAT AAAATTAATT ACGATAGCGT AACAGGACA CGTTTATAGG TTGCGAAAA CACTATCATA CCGTAAGTAA

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CAGATCTTTG CGAGATGCTT ATAGACACTG TCTATAGTAG AAATCTCTTA TATGATCAGC GCAATTATCA TGATGTTAAA CATAAAAAAT TAGATAGAGT TATTTTITTA

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ATTATACATA CTAAGTACA TATTGATTG ATGATTGACA ATAACATTG ATCTTAGT CTT AGA TTA CTA CTG CAT TGG TTC TTC TAG TAG ATG ACG GTT
-F R I I V Y G L L K D V A L
-----E3L-----

2240 2250 2260 2270 2280 2290 2300 2310 2320
TTT AGC TGC ATT ATT TTT AGC ATC TCG TTT AGA TTT TCC ATC TGC CTT ATC GAA TAC TCT TCC GTC GAT GTC TAC ACA GGC ATA AAA TGT
AAA TCG ACG TAA TAA AAA TCG TAG AGC AAA TCT AAA AGG TAG ACG GAA TAG CTT ATG AGA AGG CAG CTA CAG ATG TGT CCG TAT TTT ACA
-K A A N N K A D R K S K G D A K D F V R G D I D V C A Y P T
-----E3L-----

2330 2340 2350 2360 2370 2380 2390 2400 2410
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TCC TCT CAA TGA TCG GGG TTG ACT AAG TTA TGC TTT TCT GGT TAG AGA GAA TCA ATA AAC CGT CAT GAG TAA TTA TTA CCA CTG TCC CAA
-P S N S P G V S E I R F S W D R K T I Q C Y E N I I T V P N
-----E3L-----

2420 2430 2440 2450 2460 2470 2480 2490 2500
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TCG TAG AAA GGT TAG TTA TTA AAA AAA TCG GCC TTA TTG TAG TAG TTT TCT GAA TAC TAG GAG AGA GTA ACT AAA AAG CCG CCT ATG TAG
-A D K W D I I K K A P I V D D F S K H D E R N S K E R S V D
-----E3L-----

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-D I I V D A M A D A D P K D A E T T M F W R P P I D D S S Y
-----E3L-----

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GTG GTA TCG TGA TGC AAC TTC TAG CAT GTC TCG AAA TTA TTG AAG AGC GAA GAG GTA TAA TTC AAC AGA TCA ATC AAC ACG TCG TCA TCG
-V M A S R O L D Y L A K N V E R K E M N L Q E T L Q A A T A
-----E3L-----

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-----E3L-----

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15/15

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4430
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- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
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(54) Title: IMMUNIZING AGAINST HIV INFECTION

(57) Abstract: A virus neutralizing level of antibodies to a primary HIV isolate is generated in a host by a prime-boost administration of antigens. The primary antigen is a DNA molecule encoding an envelop glycoprotein of a primary isolate of HIV-1 while the boosting antigen is either a non-infectious, non-replicating HIV-like particle having the envelope glycoprotein of a primary isolate of HIV-1 or an attenuated viral vector expressing an envelope glycoprotein of a primary isolate of HIV-1.

INTERNATIONAL SEARCH REPORT

International Application No

PC/CA 01/00577

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/21 A61K39/39 C12N15/86 C07K14/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	CAVER T E ET AL: "A novel vaccine regimen utilizing DNA, vaccinia virus and protein immunizations for HIV-1 envelope presentation" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 17, no. 11-12, March 1999 (1999-03), pages 1567-1572, XP004158286 ISSN: 0264-410X the whole document	1,8,18, 19
Y		2-7
A		9-17



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

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- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
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Date of the actual completion of the international search

13 December 2001

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 01/00577

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 99 31250 A (ROVINSKI BENJAMIN ;CONNAUGHT LAB (CA); CAO SHI XIAN (CA); YAO FEI) 24 June 1999 (1999-06-24) cited in the application the whole document	18-22, 27-29
Y	see p. 12 line 2: BX08 ---	2-7
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